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RESEARCH SECTION

Long-term toxicity study of Black PN in mice (*J. J.-P. Drake, K. R. Butterworth, I. F. Gaunt and P. Grasso*) 503

Short-term study in the rat on two caramels produced by variations of the 'ammonia process' (*I. F. Gaunt, A. G. Lloyd, P. Grasso, S. D. Gangolli and K. R. Butterworth*) 509

Long-term toxicity study in the rat on a caramel produced by the 'half-open, half-closed pan' ammonia process (*J. G. Evans, K. R. Butterworth, I. F. Gaunt and P. Grasso*) 523

Long-term effects of calcium carrageenan in rats—I. Effects on reproduction (*T. F. X. Collins, T. N. Black and J. H. Prew*) 533

Long-term effects of calcium carrageenan in rats—II. Effects on foetal development (*T. F. X. Collins, T. N. Black and J. H. Prew*) 539

Some observations on the determination of monomer residues in foods (*J. C. Chudy and N. T. Crosby*) 547

Citrinin mycotoxicosis in the guinea-pig (*H. L. Thacker, W. W. Carlton and G. A. Sansing*) 553

Ochratoxin A mycotoxicosis in the guinea-pig (*H. L. Thacker and W. W. Carlton*) 563

Acute oral toxicity of ethylidene gyromitrin in rabbits, rats and chickens (*S. M. Mäkinen, M. Kreula and M. Kauppi*) 575

Toxicity of a moniliformin-producing strain of *Fusarium moniliforme* var. *subglutinans* isolated from maize (*N. P. J. Kriek, W. F. O. Marasas, P. S. Steyn, S. J. van Rensburg and M. Steyn*) 579

Leaching of radionuclides from neutron-activated talc in serum and in dilute hydrochloric acid (*C. L. Wilkerson, A. P. Wehner and L. A. Rancitelli*) 589

The absorption of *p*-toluenediamine by the skin of rats and dogs (*R. Hruby*) 595

Non-carcinogenicity of hair dyes: lifetime percutaneous applications in mice and rabbits (*F. G. Stenbäck, J. C. Rowland and L. A. Russell*) 601

Continued on inside back cover

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Subject/Author index to Volume 14, 1976, inserted loose in this issue

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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

The metabolism and toxicity of safrole and estragole (p. 645); The micronucleus test (p. 646).

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

COLOURING MATTERS: Brown FK mutagenic in TA1538 only (p. 649)—AGRICULTURAL CHEMICALS: Long-term effects of ethylene thiourea (p. 649); Inhaling pentachlorophenol (p. 650)—THE CHEMICAL ENVIRONMENT: Lead from kettles (p. 650); Carcinogenicity of metals (p. 651); Benzene leukaemia detailed (p. 652); Methylene chloride and ethanol synergism (p. 652)—NATURAL PRODUCTS: Congeners and alcohol metabolism (p. 653); Wool dust and the lungs (p. 653)—COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS: Genetic damage from *p*-aminobenzoic acid? (p. 654); Beauty and the beast (p. 654)—CANCER RESEARCH: Gastric cancer and nitrosamine formation (p. 655); Oral cancer in indulgent ladies (p. 655).

*These items have been contributed by the staff of the British Industrial Biological Research Association. Comments on them will be welcomed and should be sent to the Assistant Editor at BIBRA.

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FORTHCOMING PAPERS

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

Effect of amaranth, Ponceau 4R and/or vitamin A on enzyme activities of the rat liver. By D. Holmberg.

Histological examination of perinatal eye development in the rat after feeding sodium cyclamate and sodium saccharin during pregnancy. By G. Luckhaus and L. Machemer.

Formation of methylnitrosocyanamide from methylguanidine and sodium nitrite in acidic solution. By Y. Masuda, K. Shimamura and H. Endo.

Pesticide-induced modification of hepatobiliary function: hexachlorobenzene, DDT and toxaphene. By H. M. Mehendale.

Induction par le lindane des monoxygenases microsomales du foie chez le rat: Effets d'un régime hypocalcique. Par M. A. Pélissier, F. Faudemay, Ph. Manchon et R. Albrecht.

Transfer of polychlorinated biphenyls to the foetuses and offspring of mice. By Y. Masuda, R. Kagawa, S. Tokudome and M. Kuratsune.

Mycotoxicological investigations on Zambian maize. By W. F. O. Marasas, N. P. J. Kriek, M. Steyn, S. J. van Rensburg and D. J. van Schalkwyk.

Is a mixture of polychlorinated dibenzofurans an inducer of hepatic porphyria? By S. Oishi and K. Hiraga. (Short paper)

The estimation of aflatoxin M_1 in milk using a two-dimensional thin-layer chromatographic method suitable for survey work. By D. S. P. Patterson, E. M. Glancy and B. A. Roberts. (Short paper)

The biological effects of talc in the experimental animal: A literature review. By G. H. Lord. (Review paper)

Role of sultone contaminants in an outbreak of allergic contact dermatitis caused by alkyl ethoxysulphates: A review. By W. E. Lindup and P. T. Nowell. (Review paper)

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Research Section

LONG-TERM TOXICITY STUDY OF BLACK PN IN MICE

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(*Received 16 March 1977*)

Abstract—Groups of 30 male and 30 female mice were given diets containing 0·1, 0·25, 0·5 or 1·0% Black PN for 80 wk, with groups of 60 mice of each sex as controls. There were no dose-related effects on body-weight gain, haematology or organ weights. The incidence of histopathological findings, including tumours, was not altered by the feeding of Black PN. It is concluded that the addition of Black PN to the diet of mice at levels up to 1·0% (equivalent to approximately 1300 mg/kg/day) for 80 wk does not elicit any long-term toxic or carcinogenic effects.

INTRODUCTION

The food colouring Black PN (Brilliant Black BN; Brilliant Black PN; C.I. Food Black 1; C.I. (1971) no. 28440) is the tetrasodium salt of 8-acetamido-2-(7-sulpho-4-*p*-sulphophenylazo-1-naphthylazo)-1-naphthol-3,5-disulphonic acid.

It is one of the colourings permitted for use in the UK under the Colouring Matter in Food Regulations 1973 (Statutory Instrument 1973 no. 1340) and is approved for use in the EEC countries (EEC no. E151), but it is not permitted in the USA. Although it is listed in the current EEC Directive on Colouring Matters (European Economic Community, 1962) in Annex I (Colours deemed acceptable for an unlimited period), a recent report by the EEC Scientific Committee for Food (European Economic Community, 1975) classified Black PN among those colourings that are toxicologically acceptable subject to the availability of further biological data before 31 December 1978. The studies requested were metabolic investigations in several species, including man if possible, and reproductive experiments, including embryotoxicity and teratology. This Committee's estimated Acceptable Daily Intake for the colouring (0–0·75 mg/kg body weight) was considerably lower than the temporary value of 0–2·5 mg/kg established by the Joint FAO/WHO Expert Committee on Food Additives (1975), apparently on account of additional biological data made available after June 1974.

Feeding studies in pigs revealed no adverse effects, but there was a distinct blackening of the faeces (Gaunt, Colley, Creasey & Grasso, 1969). Metabolic breakdown appears to be more complete in the rat, since incubation for 19 hr with rat caecal content revealed ready degradation to colourless compounds (R. Walker, unpublished observation 1969). This was in agreement with the finding that orally administered Black PN was largely metabolized in the caecum of rats (Ryan & Welling, 1970). The chief urinary metabolite was sulphanilic acid, with smaller amounts of 4-acetamido-1-naphthylamine-6- or -7-sulphonic acid. These metabolites, together with other naphthyl derivatives and trace amounts of Black PN itself, were found in the faeces. After oral doses of Black PN, the only urinary metabolite identified in man was sulphanilic acid. Since faecal examination was not carried out, the fate of the two naphthol moieties is unknown.

The acute toxicity has been investigated in both rats and mice. After ip injection, the LD_{50} ranged from 1·1 g/kg (Gaunt, Farmer, Grasso & Gangolli, 1967) to greater than 2 g/kg (Deutsche Forschungsgemeinschaft–Farbstoff-Kommission, 1957) in rats and from 0·5 to 1 g/kg in mice (Gaunt *et al.* 1967). The LD_{50} after iv injection in rats was 2·5 g/kg (Deutsche Forschungsgemeinschaft–Farbstoff–Kommission, 1957). However, oral doses of 5 g/kg in rats and 2 g/kg in mice were not lethal (Gaunt *et al.* 1967) and an oral LD_{50} value above 5 g/kg has been recorded for mice (Deutsche Forschungsgemeinschaft–Farbstoff-Kommission, 1957).

Short-term (90-day) studies established no-untoward-effect levels of 500 mg/kg/day in rats (Gaunt *et al.* 1967) and 100 mg/kg/day in pigs (Gaunt *et al.* 1969).

Early long-term studies in rats (Deutsche Forschungsgemeinschaft–Farbstoff-Kommission, 1957), while inadequate by modern standards with respect to the number of animals used and the parameters studied, revealed no evidence of a carcinogenic effect when the colouring was included in the diet at a level of 0·1% for 410 days, or in the drinking-water at a level of 0·5% for 384 or 502 days. Observation periods in each case considerably exceeded the duration of feeding. More recently, no significant adverse effects were noted when the colouring was included in the diet of rats at levels up to 1% for 2 yr, providing intakes up to approximately 500 mg/kg/day (Gaunt, Carpanini, Grasso, Kiss & Gangolli, 1972).

This paper presents the results of an 80-wk feeding study in mice. This work, undertaken as part of the BIBRA safety evaluation programme, was designed to fulfil the requirement for information on the effects of prolonged administration of Black PN to a second rodent species.

EXPERIMENTAL

Materials. Black PN was supplied through the Chemical Industries Association and complied with

the following specification of the British Standards Institution (1968): Composition, essentially tetrasodium 8-acetamido-2-(7-sulpho-4-p-sulphophenylazo-1-naphthylazo)-1-naphthol-3,5-disulphonate; dye content*, min. 82%; subsidiary dyes*, max 4%; matter volatile at 135°C*, max 10%; matter insoluble in water*, max 0·1%; matter soluble in diisopropyl ether*, max 0·2%; chloride and sulphate (as sodium salts)*, max 8·0%; copper*, max 10 ppm; arsenic*, max 1 ppm; lead*, max 10 ppm; heavy metals (as sulphides)*, producing a colour no more intense than that of the reference standard.

Animals and diet. Mice of the CFW strain obtained from a specified-pathogen-free colony were given unlimited access to Oxoid pasteurized breeding diet and water. They were caged in groups of 15 in a room maintained at 21 ± 1°C with a relative humidity of 50–60%.

Experimental design and conduct. Groups of 30 male and 30 female mice were fed diets containing 0·1, 0·25, 0·5 or 1·0% Black PN for 80 wk. Groups of 60 male and 60 female mice with similar ranges of body weight served as controls and were fed diet containing no additive. The general condition and behaviour of the animals were observed frequently and any mouse that showed signs of ill-health was isolated, to be returned to its cage on recovery or to be killed if its condition deteriorated. Autopsies were conducted on all mice that died or were killed because of ill-health during the experiment, unless this was prevented by autolysis. The mice were weighed at the start of the experiment, at wk 3 and then at intervals of 2 wk until wk 73 of the experiment.

Blood samples were taken at wk 28 and 55 from the caudal vein of ten males and ten females from the control group and from the groups given the 0·5 and 1·0% dietary levels. At 80 wk, blood samples were collected from the aorta of all surviving mice during the autopsy. The samples were examined for haemoglobin concentration and packed cell volume, as well as for counts of erythrocytes and leucocytes. In addition, the methaemoglobin concentrations were determined in the samples collected at 80 wk. Preparations for counting the reticulocytes and the different types of leucocytes were made but, in the absence of consistent effects on the other measurements, these counts were not carried out.

*By methods of analysis described in BS 3210:1960 (Methods for the Analysis of Water-soluble Coal-tar Dyes Permitted for Use in Foods).

During wk 28, urine samples were collected over a 6-hr period from three groups of five mice of each sex from the controls and the groups on the two highest dietary levels (0·5 and 1·0%) of Black PN. These samples were examined for protein, reducing substances, bile salts and blood as well as for colour, pH and microscopic constituents.

The animals were killed by exsanguination from the aorta under sodium pentobarbitone anaesthesia following an overnight period without food. At autopsy, macroscopic abnormalities were recorded and the brain, heart, liver, spleen, kidneys, adrenal glands and gonads were weighed. Samples of these organs and of salivary glands, pituitary, thyroid, thymus, various lymph nodes, pancreas, urinary bladder, lungs, stomach, duodenum, ileum, colon, caecum, rectum, striped muscle (hind limb), spinal cord, uterus, aortic arch and any other tissue that appeared abnormal were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin. All tissues from the control mice and from those fed diet containing 1% Black PN were examined histologically. At the lower dose levels, the examination was confined to the liver, kidney and any tissues seen to be abnormal at autopsy.

RESULTS

The ingestion of Black PN had no effect on the condition or behaviour of the animals. There were deaths in all groups during the course of the study but at no time were there any statistically significant differences between the number of deaths in the control mice and those given Black PN (Table 1). Throughout the study the body weights of mice of both sexes were similar in all groups (Table 2). No abnormal constituents were detected in the urine from the control or treated mice.

The haematological examinations revealed only inconsistent isolated changes of statistical significance (Table 3). At wk 28 the haemoglobin concentrations and red blood cell counts were lower in female mice fed diet containing 0·5 or 1·0% Black PN than in the controls. There were no comparable findings in the males. The total white cell count of the male animals fed 1·0% Black PN in the diet was higher than

Table 1. *Cumulative death rate in mice fed diets containing 0–1% Black PN for 80 wk*

Wk no.	Dietary level (%)	Total no. of deaths									
		Males					Females				
		0	0·1	0·25	0·5	1·0	0	0·1	0·25	0·5	1·0
24		3	1	0	3	0	1	0	0	0	0
48		8	1	3	5	3	8	1	3	3	2
64		11	4	7	9	5	15	9	9	6	7
72		16	6	9	13	6	24	12	13	12	10
78		20	10	10	16	7	28	19	13	15	12

The figures represent the total number of mice dead or killed *in extremis* from groups of 30 (treated) or 60 (controls) of each sex. Deaths in the treated mice did not differ significantly ($P = 0·05$ by chi-square test) from those in the appropriate control group. No animals died before wk 15 of the study.

Table 2. *Mean body weights of mice fed diets containing 0–1% Black PN for 80 wk*

Wk no.	Dietary level (%)	Mean body weights (g) of Males					Females				
		0	0·1	0·25	0·5	1·0	0	0·1	0·25	0·5	1·0
0*		21	22	21	22	22	18	18	18	18	19
15		38	35	37	37	38	28	29	28	28	28
39		41	39	40	39	40	32	33	32	33	32
57		43	40	40	37	41	35	34	32	33	33
73		42	41	41	38	42	36	35	33	35	34

*Value on first day of feeding.

The figures represent the mean body weights for all surviving mice (initially 60 in control and 30 in treated groups). Those for the mice did not differ significantly ($P > 0.05$ by Student's t test) from those of the appropriate control.

that of the controls at this time, but there was no comparable change in this measurement in the females, or in either sex at any other time. At wk 55, the haemoglobin concentrations of male animals fed 0·5% of the colouring in the diet were significantly ($P < 0.05$) lower than the control values. However, the corresponding value at the higher dietary level was not affected and there were no differences from the control value in the females. Also at wk 55, the erythrocyte counts of females fed 1·0% Black PN were lower ($P < 0.01$) than those of the controls, but this finding was again isolated. There were no statistically significant differences between the control and test samples taken at wk 80.

There were only scattered differences in mean organ weights between treated and control animals. A lower brain weight in females, compared with the control value, was the only difference affecting animals fed 1% Black PN. This difference, which did not occur in the males, was only marginally significant and there was no significant difference when the weights were expressed relative to body weight (Table 4). By contrast, the relative brain weight of females fed 0·25% Black PN was higher than the control figure. Liver weights of female but not of male mice fed 0·25% Black PN were lower than control values, but again this was an isolated finding and there were no significant differences in relative liver weights. Kidney weights of male animals only were significantly lower than control values at the two lowest levels of treatment (0·1 and 0·25%), but a significant difference in relative kidney weights of males occurred only at the 0·5% level, at which a higher value was recorded for the treated mice. The only other signifi-

Table 3. *Results of haematological examinations of mice fed diets containing 0–1% Black PN for 80 wk*

Sex and dietary level (%)	No. of mice examined	Hb (g/100 ml)	Met Hb (% of Hb)	PCV (%)	RBC (10^6/mm^3)	Total leucocytes (10^3/mm^3)
			Wk 28			
Male						
0	10	14·2	—	43	7·51	11·4
0·5	10	14·4	—	48	8·05	12·4
1·0	10	15·5	—	47	8·04	14·8*
Female						
0	10	15·9	—	49	9·01	14·8
0·5	10	14·0**	—	48	7·54**	12·1
1·0	10	13·1**	—	48	7·41**	12·6
			Wk 80			
Male						
0	39	11·9	3·61	35	6·67	4·5
0·1	15	11·9	4·51	36	6·85	4·2
0·25	18	11·5	4·90	35	6·67	3·2
0·5	10	11·7	3·86	34	6·62	5·4
1·0	17	12·0	3·81	35	6·90	3·8
Female						
0	27	13·5	6·03	39	7·32	5·5
0·1	10	13·0	4·68	40	7·64	5.7
0·25	10	14·1	7·39	42	8·21	5·4
0·5	13	13·3	4·89	41	7·87	3·9
1·0	15	14·4	5·67	42	8·09	3·8

Hb = Haemoglobin MetHb = Methaemoglobin PCV = Packed cell volume RBC = Red blood cells

The figures are means for the numbers of mice shown and those marked with asterisks differ significantly (Student's t test) from those of the controls: *$P < 0.05$; **$P < 0.01$.

Table 4. *Relative organ weights of mice fed diets containing 0–1% Black PN for 80 wk*

Dietary level (%)	No. of mice examined	Relative organ weight (g/100 g body weight)							Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Adrenals†	Gonads‡	
					Males				
0	40	1·07	0·64	5·47	0·38	1·67	27·5	0·45	35
0·1	20	1·21	0·59	5·84	0·45	1·50	27·8	0·41	36
0·25	20	1·15	0·63	5·88	0·44	1·62	27·1	0·45	33
0·5	12	1·32	0·71*	5·80	0·51	1·77*	29·6	0·49	32
1·0	23	1·28	0·63	5·29	0·39	1·60	28·5	0·48	35
					Females				
0	32	1·53	0·57	5·80	0·51	1·46	40·6	84·2	29
0·1	11	1·53	0·61	5·87	0·56	1·56	37·8	84·8	28
0·25	17	1·72*	0·64*	5·29	0·41	1·51	45·9	100·8	26
0·5	15	1·55	0·56	5·43	0·54	1·41	42·8	101·2	27
1.0	18	1·53	0·53	5·27	0·47	1·40	42·3	87·8	30

†Weights are expressed in mg/100 g body weight.
‡Weights of female gonads only are expressed in mg/100 g body weight.
Values are means for the numbers of mice shown and those marked with an asterisk differ significantly (Student's *t* test) from those of controls: $^{*}P < 0.05$.

cant differences occurred in the relative heart weights, which were raised in male mice fed 0·5% and in females fed 0·25% Black PN.

The incidence of histological findings was similar in all groups of mice, including the controls (Table 5). Similarly, most of the tumours in the study occurred with either a comparable or a greater incidence in the control groups than in the treated mice (Table 6). Several isolated tumours were identified in mice given the lower levels of Black PN, without comparable findings in the controls or in the highest dose group. They were a mammary fibroadenoma (in a female on 0·1%), a uterine fibromyoma (0·1%) and a squamous-cell carcinoma of the skin (female, 0·5%).

Table 5. *Incidence of histological findings (excluding tumours) in mice fed diets containing 0–1% Black PN for 80 wk*

Tissue and histological finding	No. of mice affected: Males					Females				
Dietary level (%)...	0	0·1	0·25	0·5	1·0	0	0·1	0·25	0·5	1·0
No. of mice examined...	54	27	28	26	29	58	28	28	28	29
Lung										
Chronic inflammatory infiltration	10	10	7	9	7	9	9	6	5	5
Congestion	1	1	0	2	1	3	3	0	1	1
Liver										
Abscess	0	1	0	1	0	1	0	0	0	0
Degeneration (focal)	0	0	2	0	0	3	0	0	1	0
Macrophages (focal aggregations)	0	2	1	0	0	0	0	0	0	0
Cysts	1	0	0	0	0	0	1	0	0	0
Kidney										
Glomerulonephrosis	2	0	5	1	1	2	1	0	0	0
Pyelonephritis	1	0	0	0	0	0	0	0	0	0
Lymphoid hyperplasia	1	1	1	0	1	0	0	0	0	0
Urethra										
Chronic inflammation	1	1	2	0	0	0	0	0	0	0
Testes										
Atrophy	1	0	0	1	0	—	—	—	—	—
Ovaries										
Follicular cyst	—	—	—	—	—	3	1	1	0	5
Uterus										
Cystic	—	—	—	—	—	1	0	0	2	2
Lymphoid tissue										
Reactive hyperplasia in										
Spleen	2	0	2	1	1	0	1	1	0	0
Thymus	0	0	0	0	1	2	0	1	0	0
Lymph nodes	2	1	0	0	0	1	3	1	1	0

The figures indicate the incidence of the finding among the numbers of mice shown and those for the treated groups did not differ significantly ($P > 0.05$ by chi-square test) from those of the controls.

Table 6. *Incidence of tumours in mice fed diets containing 0–1% Black PN for 80 wk*

Tissue and finding	Dietary level (%)... No. of mice examined...	Males 0 54	Males 0·1 27	Males 0·25 28	Males 0·5 26	Males 1·0 29	Females 0 58	Females 0·1 28	Females 0·25 28	Females 0·5 28	Females 1·0 29
Lung											
Adenoma		9	1	8	5	7	14	6	3	5	6
Mammary tissue											
Fibroadenoma		—	—	—	—	—	0	1	0	0	0
Carcinoma		—	—	—	—	—	1	0	1	0	1
Adenoma		—	—	—	—	—	20	13	12	9	9
Uterus											
Fibromyoma		—	—	—	—	—	0	1	0	0	0
Skin											
Squamous cell carcinoma		0	0	0	0	1	0	0	0	1	0
Subcutaneous tissue											
Fibroma		1	0	0	0	0	0	0	0	0	0
Ovary											
Carcinoma		—	—	—	—	—	1	0	0	0	0
Lymphoid tissue											
Lymphosarcoma		2	0	1	1	1	1	1	0	1	1

The figures represent the incidences of tumours in the numbers of mice shown and those for the treated groups did not differ significantly ($P > 0{\cdot}05$ by chi-square test) from those of the controls.

The only tumour found at the highest dietary level without comparable control findings was a squamous-cell carcinoma of the skin in a male mouse fed 1% Black PN.

DISCUSSION

It is unlikely that the scattered haematological findings in this study were related to treatment with Black PN. Although in females the values for haemoglobin concentration and erythrocyte count were lower than controls at wk 28, similar findings were not encountered in the males, nor did the differences increase with more prolonged treatment. It was noticeable that the packed cell volume and the leucocyte count for the female control group at wk 28 were the highest found during the whole experiment. This suggests that some degree of haemoconcentration in this group may have led to artificially high control values. It is suggested, therefore, that the observed differences may have been related to technical problems rather than to an effect of treatment.

The changes in organ weight showed no dose-related effects. Moreover the isolated changes seen at the lower levels were not found in both sexes and were not evident when expressed relative to body weight. It is considered that these random findings were not associated with Black PN treatment.

The frequency of histopathological findings in the treated animals did not differ significantly from those in the controls. Hence, no relationship was obvious between these findings and treatment with Black PN. The incidence of tumours in the mice given Black PN in general did not exceed that in animals receiving the control diet. Only in the case of adenomas of the lung and of the mammary tissue did more than one tumour of a given type occur among the treated animals of any group. Pulmonary adenomas are among the most common tumours in untreated mice (Sher, 1974) and have been observed in control mice in our laboratories with a frequency similar to that in the present study (Brantom, Gaunt & Grasso, 1973; Evans, Gaunt, Kiss & Butterworth, 1978; Hooson, Gaunt, Kiss, Grasso & Butterworth, 1975). The number of mammary tumours in the females was unusually large, but this is a frequent cause of concern in studies involving mice (Grasso & Crampton, 1972). The frequencies appeared to fall with increasing dosage of Black PN and, on this basis, there could be no question that the colouring might have increased the incidence of this tumour. A single squamous-cell carcinoma was found at the highest dose level in a male mouse, but such malignant tumours have been found in other studies in these laboratories (Evans *et al.* 1978) and have been described in untreated animals (Brown, Gaunt, Hardy, Kiss & Butterworth, 1978; Snell, 1967). Thus this single observation cannot be considered to represent an effect of treatment, but rather the normal incidence of this tumour in mice. The observation of a single fibromyoma in a female given 0·1% Black PN may be dismissed on the basis that such tumours have been detected in untreated mice (P. L. Mason, I. F. Gaunt, K. R. Butterworth and J. Hardy, unpublished data 1974). The low incidence of lymphosarcomas in treated animals was matched by a similar incidence in control animals. Since these tumours have been found in higher incidences in previous studies in mice (Brantom *et al.* 1973) no significance is attached to the marginally higher incidence in some groups of treated females when compared to the control value.

These results provide no evidence of a carcinogenic or toxic effect on the part of Black PN when given at dietary levels up to 1% (providing an intake of 1300 mg/kg/day). Therefore it is considered that the no-untoward-effect level for Black PN in this study was approximately 1300 mg/kg.

In the feeding studies performed at BIBRA (Gaunt

et al. 1967, 1969 & 1972), the range of no-untoward-effect levels was 100–1300 mg/kg/day. On the basis of these findings and incorporating the usual 100-fold safety factor, an acceptable intake for man would be in the region of 1 mg/kg/day, or 70 mg/day for an adult.

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SHORT-TERM STUDY IN THE RAT ON TWO CARAMELS PRODUCED BY VARIATIONS OF THE 'AMMONIA PROCESS'

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Abstract—Groups of 15 rats of each sex were given diet containing 4, 8 or 16% caramel produced by either an 'open-pan' or 'closed-pan' ammonia process. A group of 25 rats of each sex served as controls. There was a reduced rate of body-weight gain at all dietary levels, although in the rats given 4% caramel there was evidence that this could be attributed to dilution of the diet. In the case of the open-pan material this also applied to the rats given 8% in the diet. There were reduced haemoglobin concentrations at the highest dietary level of both caramels, although similar findings at the lower levels were inconsistent and of doubtful significance. Lymphocytopenia was present at all dietary levels and was associated with lower weights of thymus and spleen at the two higher doses. The caecum weights were higher in the treated animals than in the controls, but this was not thought to represent a toxic effect. Increased relative liver and kidney weights suggested an effect on these organs. Changes in the weights of other organs were considered to be related to differences in body weight. A no-untoward-effect level could not be established for either of the caramels.

INTRODUCTION

Among the colourings added to foods and beverages, those grouped under the generic heading of caramels occupy a special position, not least because the process of caramelization is a well-recognized feature in many aspects of domestic food preparation and the extent to which the process is allowed to occur is largely a matter of individual preference. In almost the same way, the caramels of commercial origin are derived by methods of preparation governed as much by individual manufacturing practice as by the requirement for material serving a specific end use and chosen largely on the basis of requirements for colouring or flavouring or both.

Commercial caramels differ from most food colourings in the sense that they defeat accurate chemical specification. This difficulty is underlined by the fact that caramels are defined only by their method of manufacture in the Colouring Matter in Food Regulations (Statutory Instrument 1973, no. 1340). From the standpoint of definition, they may be dark brown to black liquids or solids, differing substantially not only in chemical composition but also in physical characteristics dependent on the method of preparation. Commercial caramelization procedures utilize the controlled thermal modification of a variety of carbohydrates, either with or without a selection of chemical 'catalysts', under a range of conditions of temperature and pressure. The most used carbohydrate source is invert sugar, itself a variable mixture of glucose, fructose and sucrose, although glucose, sucrose, molasses and starch hydrolysates (or fractions thereof) may be used. In the UK, approved 'catalysts' include a range of acids, bases or salts, which may be used singly or in combination (SI 1973, no. 1340). In addition the times and temperatures of reaction are variable, the latter being in the range 100–140°C depending on whether the process is carried out under pressure or not.

The end-products of these reactions are standardized in various ways, although there is considerable reliance on colour intensity, measured in arbitrary European Brewing Convention (EBC) units.

The chemical events accompanying the thermal modification of carbohydrates, either alone or in the presence of other reactants, are complex and an extensive literature has emerged in consequence. A detailed consideration of this information is beyond the scope of the present review. It is germane, therefore, that the Joint FAO/WHO Expert Committee on Food Additives (1972a) considered a toxicological discrimination between 'caramel' formed during the normal cooking of food and the majority of commercial caramels unwarranted and judged the latter to be acceptable for use in food. An exception was made, however, in the case of caramels prepared by processes using ammonia or ammonium salts.

There is ample evidence for the chemical variability of caramels prepared by the ammonia process. Overall compositions have been described (Joint FAO/WHO Expert Committee on Food Additives, 1972b) as comprising approximately 50% digestible carbohydrate, 25% non-digestible carbohydrate and 25% melanoidins. The relative simplicity of this view will be apparent from Fig. 1, which summarizes some of the major molecular conversions believed to occur during the reaction of aldoses with amino compounds (Maillard reaction) leading to the formation of melanoidins. These must also be presumed to take place, at least in part, during the thermal modification of carbohydrates in the presence of ammonium ions (Hodge, 1953; Hough, Briggs & Stevens, 1971;

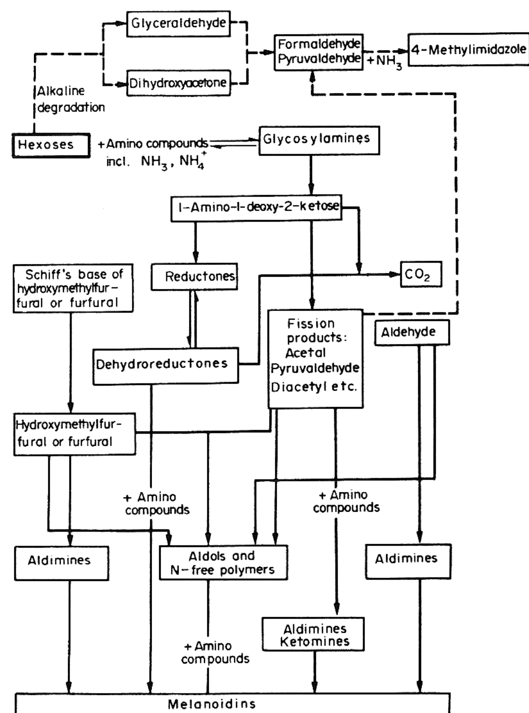

Fig. 1. The Hodge scheme for browning reactions, modified from a diagram supplied through the courtesy of Dr. C. O. Chichester. Sequences leading to the formation of 4-methylimidazole (---) are based on Carnevale (1975).

Komoto, 1962a, b; Song & Chichester, 1966 & 1967a, b; Song, Chichester & Stadtman, 1966; Wahl, 1960). Moreover, there is evidence that, in addition to the formation of low-molecular-weight volatile components, varying degrees of polymerization may occur (Sugisawa & Edo, 1964).

Many of the earlier toxicological data relating to caramels produced by ammonia processes have been reviewed in a report of the Joint FAO/WHO Expert Committee on Food Additives (1972b), and reiteration is unnecessary. On the other hand, considerable attention has been focused in recent years on the nitrogen-containing heterocyclic fractions of caramels. Concern in this area has its origins in the observations that administration of feeds supplemented with ammoniated molasses to cattle, sheep and chicks resulted in hysteria, convulsions and, occasionally, death (Bartlett & Broster, 1958; Wiggins, 1956). These effects were subsequently attributed to relatively high contents of either imidazole or pyrazine derivatives (Wiggins, 1955 & 1956; Wiggins & Wise, 1955).

The occurrence of 4-methylimidazole amongst the products of the glucose–ammonia reaction was first documented by Windaus & Knoop (1905), although subsequently some eight further substituted imidazoles have been isolated from analogous reaction mixtures (Tsuchida & Komoto, 1967). Also there is evidence that the yields of imidazole derivatives increase linearly with increases in the molar ratio of ammonia to glucose (Komoto, 1962a). By comparison, the factors influencing the formation of pyrazines in the glucose–ammonia reaction remain ill-defined, although there is tentative evidence that the pyrazine content of ammoniated molasses increases as a function of rises in both processing temperature and pressure (Wiggins, 1956). These findings are of interest in relation to the variations in the manufacture of caramels, as the pharmacological effects of imidazoles and pyrazines appear to be essentially antagonistic. Thus, in studies in rabbits, mice and chicks, Nishie, Waiss & Keyl (1969) established that the toxic manifestations of ammoniated molasses in cattle can be simulated by administering 4-methylimidazole. Moreover, while imidazole, 1-methylimidazole and 2-methylimidazole produced neurological effects in mice, similar to those caused by 4-methylimidazole, they were less potent as convulsants. Further work has established that this is the case with 4-hydroxymethylimidazole and 4-arabotetrahydroxybutylimidazole (Nishie, Waiss & Keyl, 1970). In contrast to the actions of 4-methylimidazole, mono- and dimethylpyrazines showed weak central nervous depressant activity, 2,3-dimethylpyrazine being the most potent in this regard. The majority of other pyrazines tested had no detectable biological activity.

The available information indicates that the 4-methylimidazole contents of caramels may vary in the range 50–700 ppm (Joint FAO/WHO Expert Committee on Food Additives, 1972b). Bearing this in mind and taking into account current knowledge based on animal feeding studies, the temporary acceptable daily intake for caramels produced by the ammonia process was set at 0–100 mg/kg (Joint FAO/WHO Expert Committee on Food Additives, 1972a) based on a product having a colour intensity of 20,000 EBC units and containing 200 ppm 4-methylimidazole.

The present paper describes the results of feeding rats on caramels produced by two alternative versions of the ammonia process.

EXPERIMENTAL

Materials. Two types of caramel produced by ammonia processes were used in this study. They were defined by their method of manufacture, namely caramel produced by an 'open-pan' procedure (supplied by Corn Products Ltd., Greenford, Mddx) and a caramel produced by a 'closed and open pan' process (supplied by Hay-Lambert Ltd. Uxbridge, Mddx). The latter is referred to in this report as 'closed-pan' caramel. The specifications of these materials were as follows:

Open-pan caramel: Colour, 52,000 EBC units; pH (undiluted), 4·5; extracts, 55·2 brewers' lbs/2 cwt; iron, 1·6 and copper, 0·7 ppm (w/w); nitrogen, 4·78% (w/w); sulphated ash, 0·06% (w/w).

Closed-pan caramel: Colour, 55,00 EBC units; pH (undiluted), 4·9; extract, 55·0 brewers' lbs/2 cwt; specific gravity (10% w/v solution at 15·5°C), 1·025; iron, 49·7, lead, <0·01, copper, 2·3 and arsenic, 0·01 ppm (w/w); nitrogen, 5·98% (w/w); sulphated ash, 0·80% (w/w); invert sugar, 12·01% (w/w); sucrose, 1·89% (w/w).

Animals and maintenance. Both sexes of rats of the CFE strain obtained from a specified-pathogen-free

colony (Carworth Europe, Alconbury, Huntingdon) were used in this study. They were housed in a room maintained at 20 ± 1°C with a relative humidity of 50–60%. The basal diet was Spillers' Laboratory Small Animal Diet, which, together with water, was freely available.

Conduct of experiment. Of a total of 115 male rats and 115 females, 25 rats of each sex were fed basal diet to serve as controls whilst the remainder were divided into six groups, each consisting of 15 animals of each sex. Each of these test groups was given diet containing either 4, 8 or 16% of one of the two caramels for 13 wk.

The rats were housed five to a cage and were acclimatized for 7 days before receiving the caramel diets. They were weighed 3 days before the start of the treatment, 24 hr after the onset of treatment and then weekly for the remainder of the study. The quantity of food consumed by each cage of rats was measured over the 24-hr period preceding each day of weighing.

Blood samples for haematological examinations were collected from the tail veins of eight rats of each sex from each group during wk 6 and 10. Further samples were taken from the aorta of all rats prior to the post-mortem examination at wk 13. Total and differential leucocyte counts were made. In addition, at wk 6 and 13, data on haemoglobin concentration, packed cell volume and erythrocyte and reticulocyte counts were obtained. At wk 13 the concentration of methaemoglobin was measured.

Urine was collected from twelve rats of each sex from each treated group and from 18 controls of each sex during the last week of treatment. These samples were examined for appearance and for contents of glucose, ketones, bile salts, blood and protein, and the microscopic constituents of the sediment were studied. At the same time, renal function tests were carried out, involving the measurement of the volume and specific gravity of the urine produced in a 2-hr period following a water-load of 25 ml/kg, in a 6-hr period of water deprivation and in a 4-hr period following 16 hr without water. The activity of glutamic–oxalacetic transaminase was measured in the urine collected over the 6-hr period.

The rats were killed by an ip injection of barbiturate following an overnight period without food. At autopsy, a search was made for abnormalities and the brain, pituitary, thyroid, thymus, heart, liver, spleen, kidneys, adrenal glands, gonads, caecum with its contents and lymph nodes (a standard four from the dorsal wall of the abdomen and four from the cervical region) were weighed. Samples of these tissues and of trachea, oesophagus, skeletal muscle, colon, rectum, lung, urinary bladder, uterus, aorta and any tissue that appeared to be abnormal were preserved in buffered formalin. After processing for wax embedding, 5-μm sections of these tissues were stained with haematoxylin and eosin for histological examination. This examination was made on the tissues from all control rats, from all of those given diet containing 16% caramel and from half of those given the two lower dietary levels.

Serum was separated from the blood collected at post-mortem examination and analysed for its content of urea as well as for the activities of glutamic–pyruvic and glutamic–oxalacetic transaminases.

RESULTS

Apart from a black staining of the fur and tail due to external contamination with the diet, the animals appeared to be normal. As judged visually, the faeces from the rats given high doses of caramel were less well formed than those of the control rats and were of a darker colour. The body weights of both sexes given the highest dose of either caramel were lower than those of the controls, to a statistically significant degree, throughout the study (Table 1). At the intermediate dose level there was a similar significant reduction in body weight throughout the study in the females, whereas in the males the differences from controls were statistically significant from day 22 onwards. At the lowest dietary level there were statistically significant differences during most of the study in the females. In males such differences were evident from day 22 with the closed-pan caramel and from day 36 with the open-pan material.

The overall mean food intakes (Table 1) of all treated groups were similar to or greater than those of the controls; indeed the only statistically significant differences from the control figures were higher values for the males given diet containing 16% open-pan caramel or 8% closed-pan caramel. However, consideration of the individual figures shows that all groups given diet containing 16% caramel consumed significantly less diet than the controls on the first day of treatment. A similar effect was noted in both sexes given the intermediate level of the closed-pan caramel. The intakes of caramel were high, the calculated means ranging between approximately 3 and 13·5 g/kg for the three dietary levels (Table 1).

Compared with the control values, there were no statistically significant differences in the values for packed cell volume, reticulocytes or methaemoglobin concentration (Table 2). On the other hand, there were lower values for haemoglobin concentrations and erythrocyte counts in rats treated with either caramel. This was most obvious in the male rats given the closed-pan caramel, the values for haemoglobin concentration being lower at all three dietary levels at both wk 6 and 13. In general, these reductions were associated with reduced erythrocyte counts. In the comparable female group, the haemoglobin concentration was lower than the controls at the highest dietary level at both examinations but at the intermediate level only at wk 13, and there were no statistically significant differences in the erythrocyte counts. The changes were less consistent in rats fed the open-pan caramel. The haemoglobin concentrations in the male rats were less than the controls at all three dietary levels at wk 13 but only at the intermediate dose at wk 6. In these rats, the only statistically significant reductions in erythrocyte count, compared with the controls, occurred at the two lower dose levels at wk 6. In the females the haemoglobin concentration was lower than the controls at the highest dose level at wk 6 and 13, but the erythrocyte count was lower only at wk 13. Also the latter measurement was lower than the control value at wk 6 in the rats given 8% open-pan caramel.

The outstanding feature in the leucocyte counts (Table 3) was a reduction in the number of lymphocytes, although in the females at wk 13 this was not seen with either of the caramels. In animals fed the

Table 1. *Body weight and food intake of rats given diets containing 0–16% ammonia-catalysed caramel for 13 wk*

Type of caramel and dietary level (%)	Body weight (g) on day							Food intake (g/rat/day) on day							Mean food intake (g/rat/day)	Mean caramel intake (g/kg/day)
	−3	1	8	29	50	71	92	−3	1	8	29	50	71	92		
Males																
0 (control)	116	147	189	307	383	431	462	15·7	18·8	18·7	23·1	22·9	21·4	21·5	21·8	
Open-pan																
4	115	145	188	295	363*	410*	441	14·7	18·5	19·4	22·9	22·0	22·0	20·8	21·6	2·87
8	115	145	185	289**	351**	394***	424**	16·0	17·5	20·1	23·7	23·3	21·7	22·2	22·2	6·00
16	116	138**	173***	271***	329***	369***	395***	15·5	11·6**	19·9	24·7	27·9	27·3	26·5**	24·6*	13·63
Closed-pan																
4	116	145	185	293*	361*	411*	428***	16·0	18·7	18·5	22·9	23·3	21·6	21·7	21·8	2·90
8	116	145	185	295*	357**	397***	425***	17·0	17·3*	20·3	26·7**	24·7	24·3**	23·7	23·7*	6·27
16	116	138**	168***	271***	324***	363***	389***	15·8	11·3**	18·1	26·5**	23·9	22·1	24·2	22·7	12·68
Females																
0 (control)	108	132	156	210	244	274	289	14·3	16·0	15·6	19·4	18·4	17·5	14·5	17·5	
Open-pan																
4	106	127	148*	193**	221***	248***	264***	14·2	16·0	14·6	18·6	18·4	16·6	16·0	16·9	3·34
8	106	130	150*	200**	226***	252***	264***	13·7	15·1	14·3	19·5	19·3	18·3	17·0**	17·8	6·68
16	110	129	150*	195**	223***	246***	262***	14·5	9·7**	14·8	17·8	18·2	15·6*	17·6*	17·7	13·88
Closed-pan																
4	106	129	149*	199**	234*	259**	274**	13·9	15·3	15·0	16·6	17·9	16·1*	15·9	16·8	3·21
8	108	128*	147**	195***	224***	248***	259***	14·2	12·3**	14·1	19·4	18·5	17·6	17·1*	17·4	6·76
16	108	124**	148*	194***	223***	249***	261***	14·8	8·0**	14·9	20·3	19·7	18·1	18·2*	18·0	13·83

The figures for body weight are means for groups of 25 control and 15 treated rats and those for food intake are means for five cages of five control rats and three cages of five treated rats. The figures marked with asterisks differ significantly from those of the appropriate controls: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$. (The statistical methods used were Student's *t* test for body weight and mean food intake and the Lord (1947) test for the individual food intakes.) Mean intakes of caramel are derived from weekly figures calculated from the body-weight and food-intake data.

Table 2. *Erythrocyte measurements for rats given diets containing 0–16% ammonia-catalysed caramel for 6 or 13 wk*

Type of caramel and dietary level (%)	Erythrocyte values for: Males — Hb (g/100 ml)	MetHb (% of Hb)	PCV (%)	RBC ($10^6/mm^3$)	Retics (% of RBC)	Females — Hb (g/100 ml)	MetHb (% of Hb)	PCV (%)	RBC ($10^6/mm^3$)	Retics (% of RBC)
					Wk 6					
0 (control)	15·6	—	47	8·28	1·5	15·3	—	46	7·88	1·6
Open-pan										
4	14·9	—	46	7·60**	1·5	14·5	—	46	7·72	2·2
8	14·7	—	46	7·69**	1·3	14·7	—	47	7·29*	1·8
16	15·1	—	48	8·02	1·1	13·9***	—	47	7·64	0·9
Closed-pan										
4	14·8*	—	49	8·31	2·2	15·0	—	47	8·12	1·3
8	14·8*	—	48	7·73**	1·6	15·3	—	48	7·36	1·6
16	13·8***	—	48	7·69***	1·4	13·5***	—	46	7·58	2·0
					Wk 13					
0 (control)	16·1	3·3	46	8·97	0·8	14·8	2·3	43	7·86	1·1
Open-pan										
4	15·2*	3·0	45	8·31	0·9	14·6	2·5	43	7·93	1·1
8	15·0**	2·6	45	8·27	0·9	14·2	2·6	43	7·52	1·7
16	15·2*	3·3	45	8·16	0·8	13·7*	2·4	43	7·31***	1·5
Closed-pan										
4	15·0*	3·0	45	8·17**	0·9	14·4	2·6	43	7·47	1·2
8	14·5**	3·6	44	7·99***	1·1	13·9*	2·7	43	7·62	1·7
16	14·8**	2·8	45	7·99***	0·9	14·0*	2·3	42	7·54	1·5

Hb = Haemoglobin MetHb = Methaemoglobin PCV = Pack cell volume RBC = Red blood cells Retics = Reticulocytes

The figures are means for groups of eight rats (12 controls) at wk 6 and for groups of 15 (25 controls) at wk 13 and those marked with asterisks differ significantly (Student's *t* test) from the appropriate control value: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 3. *Leucocyte studies in rats given diets containing 0–16% ammonia-catalysed caramel for 6, 10 or 13 wk*

Type of caramel and dietary level (%)	Numbers of leucocytes ($10^3/mm^3$)									
	Males					Females				
	Total	N	E	L	M	Total	N	E	L	M
					Wk 6					
0 (control)	17·7	2·0	0·4	14·6	0·7	14·4	1·4	0·3	12·1	0·5
Open-pan										
4	10·3***	3·0**	0·3	6·3***	0·7	13·8	2·8***	0·4	10·0*	0·6
8	10·5***	3·2*	0·3	6·6***	0·4	13·0	3·7***	0·5	8·2**	0·7
16	8·3***	2·9*	0·2	4·9***	0·3	9·7***	2·8***	0·5	6·1**	0·4
Closed-pan										
4	10·5***	2·6	0·2	7·2***	0·5	13·7	3·2***	0·5	9·5**	0·6
8	12·5***	3·9***	0·3	7·6***	0·7	11·4**	3·1***	0·4	7·5***	0·4
16	8·3***	2·8***	0·3	4·9***	0·3	10·7***	2·6***	0·4	7·3***	0·4
					Wk 10					
0 (control)	16·9	1·9	0·5	13·7	0·9	14·0	1·4	0·3	11·8	0·5
Open-pan										
4	11·3***	2·8	0·3	7·4***	0·9	10·1*	2·1	0·5	6·8**	0·8
8	10·7***	3·5*	0·3	6·2***	0·7	10·9	3·2***	0·6	6·5**	0·5
16	12·6**	4·5**	0·7	6·3***	1·1	9·3*	2·8	0·5	5·6***	0·6
Closed-pan										
4	12·8**	4·5**	0·3	7·0***	1·1	10·7	2·8*	0·3	7·1**	0·6
8	12·8**	4·7**	0·5	6·8***	0·9	10·1	2·3**	0·4	6·6**	0·8
16	13·6	5·8**	0·5	6·3***	1·1	10·6	3·6**	0·4	5·8***	0·8
					Wk 13					
0 (control)	7·4	1·2	0·2	5·7	0·3	3·7	0·6	0·1	2·8	0·2
Open-pan										
4	6·7	1·2	0·2	5·0	0·4	4·0	0·6	0·2	3·0	0·2
8	5·6**	1·1	0·1	4·2**	0·2	4·9	0·8	0·1	3·7	0·2
16	5·0***	1·1	0·1	3·5***	0·2	4·0	0·8	0·1	2·8	0·3
Closed-pan										
4	5·8*	1·0	0·2	4·4**	0·2	4·3	0·7	0·1	3·3	0·2
8	5·8*	1·6	0·2	3·8***	0·2	3·7	0·7	0·1	2·7	0·2
16	4·9***	1·5	0·2	2·9***	0·3	4·2	0·8	0·1	3·0	0·3

N = Neutrophils E = Eosinophils L = Lymphocytes M = Monocytes

Numbers of the different types of leucocytes were calculated from the total values and the proportions derived from examination of a blood smear. The figures are means for groups of eight rats (12 controls) at wk 6 and 10 and for groups of 15 (25 controls) at wk 13 and those marked with asterisks differ significantly (Student's t test) from the appropriate control value: $^*P < 0·05$; $^{**}P < 0·01$; $^{***}P < 0·001$.

open-pan caramel, values for the lymphocyte counts were lower than those of the controls at all dietary levels in both sexes at wk 6 and 10 and at the two top dietary levels in males at wk 13. These differences were statistically significant. The findings were similar with the closed-pan caramel, with the addition that the count in males at the lowest dietary level was also reduced compared with the controls at wk 13. In general these differences in lymphocyte count were accompanied by reductions in the total leucocyte counts, but since there were frequently increases in the numbers of the other types of leucocytes, particularly of neutrophilic polymorphonuclear cells, this was not always so. Because of the increases in other cell types, the extent of the lowering of the total white cell counts was less than that of the lymphocytes.

Apart from a dark colouring in all treated animals, no abnormal urinary constituents were found in any of the rats. The colouring was not seen in urine collected from the bladder at autopsy. The volumes of urine excreted during a 6-hr period without water or in the 2 hr after a water load were lower than the control values (Table 4). The latter differences were accompanied by higher values for specific gravity.

There were scattered statistically significant differences from controls in the serum levels of urea and the transaminases. However, the only measurements that were higher than those of the controls were the urea concentrations in males given 16% of either caramel ($P < 0·05$).

There were many statistically significant differences from the controls in the analysis of organ weights and relative organ weights (Tables 5 & 6). In general the heart weights of rats given 16% caramel were lower than those of the controls, although the difference was statistically significant only in males given the open-pan material. However, when expressed relative to body weight the heart weights were, in the main, higher than the control values. The situation regarding liver weights differed in the two sexes. In males the liver weights of all treated groups were lower than those of the controls but to a statistically significant

Table 4. *Results of renal concentration and dilution tests and urinary GOT determination in rats given diets containing 0–16% ammonia-catalysed caramel for 12 wk*

Type of caramel and dietary level (%)	Concentration test				Dilution test (2 hr)		
	Specific gravity		Volume (ml)				
	0–6 hr	16–20 hr	0–6 hr	16–20 hr	Specific gravity	Volume (ml)	GOT
			Males				
0 (control	1·066	1·087	2·0	0·8	1·009	5·5	3·1
Open-pan							
4	1·070	1·081	1·1*	0·7	1·010	4·9	2·7
8	1·074	1·089	1·3	0·7	1·015	3·1*	3·5
16	1·069	1·082	0·6**	0·9	1·010	4·0	5·0
Closed-pan							
4	1·065	1·084	1·4	1·0	1·007	5·2	2·1
8	1·069	1·083	1·4	0·7	1·011	4·3	2·0
16	1·074	1·086	1·1*	0·9	1·013	3·2*	2·6
			Females				
0 (control)	1·059	1·085	1·0	0·4	1·006	5·2	3·9
Open-pan							
4	1·042	1·081	0·9	0·5	1·008	4·0	2·3
8	1·072	1·079	1·2	0·5	1·008	4·6	3·2
16	1·055	1·075	0·8	0·6	1·010*	4·5	4·0
Closed-pan							
4	1·056	1·083	0·9	0·5	1·010	3·9	3·3
8	1·060	1·072	0·9	0·6*	1·007	4·9	3·1
16	1·061	1·086	1·2	0·5	1·010*	3·8*	3·6

GOT = Glutamic–oxalacetic transaminase

The figures are means for groups of 12 treated rats and 18 controls, and those marked with asterisks differ significantly (Lord, 1947, range test) from the appropriate control values: $^{*}P < 0·05$; $^{**}P < 0·01$.

extent only in those given 16% open-pan caramel. On the other hand, the female liver weights tended to be increased, significantly so in those given 16% of either material and those given 8% of the open-pan sample. However, when expressed in relation to body weight, all the values were increased in comparison with the controls. This difference was statistically significant in all treated female groups, in both male groups given 16% caramel and in the males given 8% closed-pan caramel.

The weights of the spleen were lower than the control weights in both sexes given the two higher dietary levels of either caramel, but when expressed relative to body weight the values were comparable with those of the controls. The only statistically significant differences from the controls in the kidney weights were higher values in both sexes given 8% open-pan caramel. However, the differences from the controls in relative kidney weight were statistically significant in all groups given 8 or 16% caramel and in the males given 4% open-pan caramel.

Dose-related increases in caecum weights reached significance in the groups given 8 or 16% caramel. These same differences were evident when the weights were expressed relative to body weight and, additionally, there was a significant increase for the females given 4% open-pan caramel.

The weights and relative weights of the thymus of all male groups given caramel were lower than the control values. In the females, the weights were lower than those of the controls but the differences were statistically significant only for the relative values for the groups given the highest dietary level of either material and the lowest level of the closed-pan caramel. There were lower weights and relative weights for lymph nodes in the males given 16% closed-pan caramel and in the females given the same dietary level of open-pan caramel. Although there were lower thyroid weights in both sexes given the higher levels of open-pan caramel, these were not reflected in the relative weights. In the remaining organs there were higher values for the relative weights with no significant differences in the absolute weights.

The post-mortem examination revealed obvious enlargement of the caecum in most treated groups. In addition, small free-living nematodes were present in the caecum and colon of some of the rats. These were of the non-pathogenic species *Syphacia obvolata* and were found in three female rats (two controls and one given 4% open-pan caramel). They were found more frequently in the males, with 16% of the controls affected and incidences of 20 and 26% in rats given the lowest dietary level of open- and closed-pan caramels, respectively. At the intermediate dietary levels, 13% of the animals were affected, whilst at the highest level no nematodes were found.

The histological examination revealed minor changes in the lungs, such as alveolar collapse together with peribronchial and perivascular cuffing with leucocytes. In addition there was renal tubular dilatation or minor renal tubular atrophy. However, these lesions were of similar severity and distributed with a similar frequency in all groups including the controls. A careful and extensive examination of the spleen, lymph nodes, thymus and other lymphoid tissue did not reveal any treatment-related changes.

Table 5. *Organ weights of rats given diets containing 0–16% ammonia-catalysed caramel for 13 wk*

Type of caramel and dietary level (%)	Organ weight (g)												Terminal body weight (g)
	Brain	Heart	Liver	Spleen	Kidneys	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	Thymus	Lymph nodes§	
Males													
0 (control)	1·83	1·35	12·42	0·70	3·09	3·09	64	3·52	11·5	26	0·45	170	445
Open-pan													
4	1·82	1·35	11·71	0·64	3·11	3·44	62	3·59	12·0	24	0·36**	160	420*
8	1·82	1·34	11·73	0·61**	3·30*	3·81*	65	3·77	11·9	25	0·33***	150	403***
16	1·82	1·27*	11·32*	0·53***	3·18	5·95***	58	3·60	10·3	22*	0·29***	140	370***
Closed-pan													
4	1·87	1·35	11·72	0·64	3·09	3·25	66	3·64	11·3	24	0·30***	140	416***
8	1·86	1·36	11·91	0·61**	3·32*	4·76***	65	3·68	12·7	26	0·29***	150	403***
16	1·82	1·26	11·53	0·53***	3·20	7·44***	64	3·52	11·9	23	0·23***	120***	372***
Females													
0 (contol)	1·73	0·93	6·31	0·53	1·90	2·23	70	130	12·2	22	0·28	150	285
Open-pan													
4	1·71	0·91	6·31	0·50	1·80	2·48	68	139	11·0	19	0·24	140	256***
8	1·72	0·94	6·86**	0·49*	1·99	2·88**	68	130	12·3	19*	0·22	150	258***
16	1·70	0·88	7·23***	0·45**	2·01	3·78***	69	143	12·7	17**	0·20	120**	253***
Closed-pan													
4	1·73	0·91	5·20	0·51	1·89	2·54	68	134	12·6	20	0·21	150	267**
8	1·75	0·89	6·59	0·48*	1·88	2·75**	67	133	12·4	21	0·22	130	250***
16	1·75	0·88	7·15**	0·46**	2·05	5·56***	69	131	12·4	20	0·21	130	253***

†Values for these organs are expressed in mg.
‡Values for female gonads are expressed in mg.
§Weights of eight selected lymph nodes expressed in mg.
Figures are means for groups of 25 control and 15 treated animals and those marked with asterisks differ significantly (Student's t test) from the corresponding control values: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Table 6. *Relative organ weights of rats given diets containing 0–16% ammonia-catalysed caramel for 13 wk*

Type of caramel and dietry level (%)	Relative organ weight (g/100 g body weight)											
	Brain	Heart	Liver	Spleen	Kidneys	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	Thymus	Lymph nodes§
Males												
0 (control)	0·41	0·30	2·77	0·16	0·69	0·76	14·4	0·79	2·6	5·8	99	37
Open-pan												
4	0·43	0·32*	2·78	0·15	0·73*	0·81	14·6	0·85	2·9	5·8	86	38
8	0·45	0·33**	2·91	0·15	0·82***	0·95**	16·1*	0·95**	2·9	6·2	79**	38
16	0·49	0·34**	3·01**	0·14	0·85***	1·58***	15·6	0·97***	2·8	5·8	80**	39
Closed-pan												
4	0·45	0·33*	2·81	0·15	0·68	0·78	14·8	0·88*	2·5	6·2	73***	34
8	0·46	0·34***	2·95*	0·15	0·81***	1·10***	16·2*	0·91***	3·2***	6·6	71***	37
16	0·49	0·34***	3·09***	0·14	0·86***	2·00***	17·3**	0·94***	3·2***	6·2	63***	31***
Females												
0 (control)	0·61	0·32	2·22	0·19	0·66	0·81	24·5	46	4·3	7·6	100	51
Open-pan												
4	0·67	0·36*	2·48***	0·20	0·70	1·00**	25·7	54	4·3	7·4	93	53
8	0·67	0·36*	2·64***	0·19	0·77***	1·11***	26·5	50	4·8	7·3	88	54
16	0·67	0·35*	2·85***	0·18	0·79***	1·55***	27·3*	56	5·0**	6·9	80**	45**
Closed-pan												
4	0·64	0·34	2·52***	0·19	0·67	0.94	25·6	50	4·6	7·7	79***	55
8	0·70	0·34	2·64***	0·19	0·75**	1·10**	27·7**	53	5·0**	8·2	90	53
16	0·69	0·35*	2·82***	0·18	0·81***	2·16***	27·5**	52	4·9**	7·7	84*	51

†Values for these organs are expressed in mg/100 g body weight.
‡Values for female gonads are expressed in mg/100 g body weight.
§Weights of eight selected lymph nodes expressed in mg/100 g body weight.
Figures are means for groups of 25 control and 15 treated animals and those marked with asterisks differ significantly (Student's *t* test) from the corresponding control values: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

DISCUSSION

Despite the high dose levels, providing average caramel intakes of up to 13 g/kg/day, there was no indication of behavioural abnormality in the rats during this study. There were no signs of damage to the central nervous system at post-mortem or during the histological examination. As a further investigation of possible neurological effects, M. Sharratt (unpublished observations 1971) gave single oral doses of up to 10 and 15 g/kg to mice and rabbits, respectively. No abnormalities were detected. On the basis of these observations there is no reason to associate the caramels used in this study with hysteria of the type reported by Bartlett & Broster (1958) and Wiggins (1956) in studies on ammoniated molasses.

The dose-related reductions in the body-weight gain without parallel reductions in the food intake are suggestive of a toxic effect on the part of the caramels, one possibility being a local effect on the intestine, perhaps of an irritant nature, with consequent reduction in the absorption of nutrients. Our data do not warrant more specific conclusions, except that they suggest an unpalatable effect of the diet in the early stages of the experiment.

The dietary levels were high and it is possible that the processing of the carbohydrates affects their bioavailability. The data cited by the Joint FAO/WHO Expert Committee on Food Additives (1972b) suggest that up to 50% of the carbohydrate may be in a digestible form. A reduction in body-weight gain due to the presence of non-digestible material should be reflected in the food-utilization ratios (weight gain per unit of food intake). Assuming that the lower weight gain was due to the dilution of the food, these ratios would show a reduction when based on the total food intake but would be normal if account were taken of the dilution factor. These ratios, based on the mean values for food intake and body weight cannot be accurate. Nevertheless Table 6 shows that there were considerable reductions in the utilization based on total food intake at the two higher dose levels, the reductions being most marked with the closed-pan caramel. When the food intake without the additive was considered, there were smaller reductions in the ratio at the highest dose level of either caramel but only with the closed-pan material at the intermediate dosage.

This suggests that at the lowest level of caramels used in the present study the reductions in weight gain may be attributed to dilution of the diet. At the highest level, on the other hand, the figures suggest that some other factor was influencing the weight gain. With the closed-pan material at the intermediate level there were similar effects that were not wholly attributable to dietary dilution.

Adrian (1973) discussed the activity of the soluble premelanoidin products of the Maillard reaction and showed that these materials were capable of reducing the digestibility and utilization of protein. These observations could provide a basis for the effects of the caramels.

In both sexes a lowered haemoglobin concentration was associated with the feeding of either of the caramels at 16% of the diet. There were suggestions of a similar effect at the lower levels but these were less consistent, as were the lower erythrocyte counts. The reticulocyte counts gave no indication of the cause of these reductions. If the reductions were due to haemolytic or haemorrhagic effects it would be expected that there would be a reticulocytosis in response to the erythrocyte loss. If the reduced erythrocyte counts were attributable to a marrow hypoplasia, the reticulocyte count would be expected to be lower than that of the controls, but there was no evidence of this. The anaemia could have been due to an iron deficiency related either to the reduced intake of nutrients caused by dilution of the diet or to the caramels acting to prevent the absorption of iron. However, this seems unlikely since iron-deficiency anaemia tends to be hypochromic and microcytic (Wintrobe, 1967) but calculation of the absolute values showed that those for the treated groups were within 10% of the control values. Hence, it is not possible, at present, to account for the aetiology of the anaemia.

Changes in the leucocyte counts were even more notable than the changes in the erythrocytes. These changes were first noticed as reductions in the total count, but from the differential counts it was obvious that the reductions resulted from a lymphocytopenia. This was somewhat obscured by an increase in the numbers of neutrophilic polymorphonuclear cells. There was no marked difference in the response of the lymphocytes to the two different types of caramel.

The total leucocyte counts at the termination of the experiment (13 wk) were much lower than those observed at intermediate times (Table 3). This reflects the different methods of obtaining blood, from superficial veins at wk 6 and 10 and from the aorta for

Table 6. *Food conversion ratio of rats given 0–16% ammonia-catalysed caramel for 13 wk*

Period of study (days)	Dietary level (%)..	Food conversion ratio						
		Control	Open-pan caramel			Closed-pan caramel		
		0	4	8	16	4	8	16
		Based on total food intake						
0–50		0·22	0·21	0·19	0·17	0·20	0·18	0·16
0–92		0·16	0·15	0·14	0·12	0·16	0·13	0·12
		Based on food intake without additive						
0–50		0·22	0·22	0·21	0·20	0·21	0·20	0·19
0–92		0·16	0·16	0·15	0·15	0·16	0·14	0·14

The figures are ratios (g weight gain/g food consumed) over the periods shown and are calculated from data in Table 1.

the terminal counts. Such differences are commonly observed (Gaunt, Brantom, Grasso, Creasy & Gangolli, 1972; Gaunt, Carpanini, Grasso, Kiss & Gangolli, 1972; Hardy, 1967; Schermer, 1954) and are reflected in the data on absolute numbers of different types of white cell, since the latter are derived by a calculation involving the total and differential white cell counts. Attention is drawn to these differences in this particular study because lymphocytopenia is one of the principal effects observed, but this does not affect the basic conclusions.

There is no evidence from the present study to suggest whether the lymphocytopenia was the result of reduced production or increased destruction of the cells. It does not appear to be part of a general reduction in bone-marrow activity since there was a tendency for an increase in the number of circulating polymorph cells. Further indication of the adequate capacity of the marrow was provided, for the erythroid line of cells, by the normal numbers of reticulocytes.

A severe limitation of food intake and weight gain (to 10% of normal) has resulted in reduced lymphocyte counts (Shukers & Day, 1943). This degree of dietary reduction was more severe than that encountered in the present study, however, and in addition, no selective lymphocytopenia has been encountered in this laboratory in other studies in which reductions in weight gain occurred (Carpanini, Gaunt, Kiss, Grasso & Gangolli, 1973; Gaunt, Sharratt, Grasso, Lansdown & Gangolli, 1974). A study of this phenomenon is in progress in our laboratory but, on the basis of the available evidence, it must be assumed that the lymphocytopenia resulted from the administration of the caramels.

It is likely that there is an association between the reductions in weight of the lymph nodes, thymus and spleen and the lower numbers of circulating lymphocytes. It has been estimated (Jandl, Files, Barnett & MacDonald, 1965) that approximately 50% of the cells of the rat spleen are lymphocytes. On this basis, the 12, 26 and 39% reductions in lymphocytes at wk 13 in males given the open-pan caramel might be expected to lead to 6, 13 and 19% lower spleen weights. The actual reductions were 9, 13 and 24%. The corresponding expected spleen-weight reductions for the closed-pan material were 11, 17 and 25% compared with actual reductions of 9, 13 and 24%. The degree of correlation between the expected and actual values supports the suggestion that the lower splenic weights may be related to the reduced numbers of lymphocytes. An alternative explanation of the reduced spleen weight may be the differences in body weight. Rats that had failed to grow at a normal rate because of a 12·5% restriction of their food intake (Schwartz, Tornaben & Boxill, 1973) showed differences in body weight and spleen weight similar to those of the animals given 16% caramel in the present study.

Another notable effect of both caramels was an increase in caecum weights. There are a number of possible explanations for this increase. It has been shown that various materials cause caecal enlargement by increasing the osmotic activity of the caecal contents (Leegwater, de Groot & von Kalmthout-Kuyper, 1974). The black colour of the faeces in the present experiment suggests that a considerable portion of the caramel was not absorbed in the gastro-intestinal tract. Hence there would be high concentrations of various components of caramel in the caecum, and these would possibly be capable of exerting the osmotic effects described by Leegwater *et al.* (1974). These authors considered that such increases were not of toxicological significance. This seems unlikely to represent the whole explanation, however, in view of the observation that the soluble premelanoidins can lead to caecal enlargement (Adrian, 1973).

An alternative explanation is based on the observations that high-fibre diets can lead to caecal enlargement (Hoover & Heitmann, 1972; Young, Mancharan & Young, 1969) and that the caecal microflora is associated with breakdown of cellulose (Conrad, Watts, Iacono, Kraybill & Friedmann, 1958). In this context, materials with possible biocidal effects, such as surfactants, often cause caecal enlargement (Alfredson, Stiefled, Thorp, Baten & Gray, 1951; Finnegan, Larsan, Smith, Haag, Reid & Dreyfus, 1953; Fitzhugh & Nelson, 1948). On this basis it is possible that by altering the local microflora, the administration of caramel could result in caecal enlargement through changes in the digestion of cellulose. Such a suggestion gains some support from the observation (Adrian, 1973) that the development of certain micro-organisms is modified by the premelanoidins.

The action of either of these possible mechanisms depends on the local concentration of caramel within the caecum. It is unlikely that the concentrations used in the present study would be encountered as a result of the use of caramel in food. In view of this and the fact that the caecal weights were within normal limits in rats given 4% caramel in their diet, the enlargement of the caecum is considered to be of no significance in the assessment of any hazard from caramel.

The weights of many of the organs showed changes in relation to the controls but most of these could be related to the failure to gain weight at a normal rate. Lower values for heart weight, with normal or slightly increased values when the weights are expressed relative to body weight, have been reported in rats with low body weights resulting from the feeding of reduced quantities of normal diet (Schwartz *et al.* 1973) or from the feeding of a diet containing undigestible cellulose (Feron, de Groot, Spanjers & Til, 1973). Similarly the isolated changes in pituitary, adrenal, gonad and thyroid weights may be related to the failure to gain weight at a normal rate rather than to any effect of treatment.

The lower values for liver weight are in keeping with the findings in animals given reduced quantities of food, but the increased relative values do not follow the usual pattern in such animals. Indeed Feron *et al.* (1973) consider such increases to be related to treatment. A possible effect on the liver cannot be ruled out in view of the finding of increased liver weight and hepatic necrosis in animals given premelanoidins (Adrian, 1973). However, with the caramels there was no histological evidence of hepatic damage. The work of Schwartz *et al.* (1973) and that of Feron *et al.* (1973) suggests that the increases in relative kidney weights, as seen in the present study, should be regarded as effects of treatment. However, the in-

creased kidney weights were not associated with any histopathological changes and there was no indication of any adverse changes in the renal function tests, although the higher values for blood urea may indicate some marginal kidney damage at the highest dietary level.

In this study caramels were fed at very high concentrations in the diet. This led to many changes in the measurements carried out during the study, some of which can be related to a reduced rate of growth whilst others appear to be directly associated with the treatment. In summary, the findings are that with the open-pan caramel there was a reduced rate of body-weight gain at all levels of treatment. Consideration of the food utilization ratios suggests that this effect could have been due to dilution of the diet in rats given the 8% level but that some other factor influenced the weight gain at the highest dietary level. Consistent reductions in haemoglobin, apparently related to treatment, occurred in both sexes given the diet containing 16%. Similar changes at the lower levels, seen only in males, were inconsistent and of doubtful significance. On the other hand, lymphocytopenia was found at all levels of treatment. This was associated with lower thymus weights in the males and with lower splenic weights at the two higher levels. Increased relative liver and kidney weights indicated the possibility of an effect on these organs at all levels.

Essentially similar conclusions can be drawn in the case of the closed-pan caramel, with the addition that there was an indication of effects on weight gain not attributable to dietary dilution in the rats given the diet containing 8%.

Since at all dose levels there were various findings differing from those in the control groups, it is not possible to establish a no-effect level for either of the caramels. Final conclusions on the toxicological significance of many of the findings depend on specific investigation of the nature of the effects. Some studies of this nature are underway in our laboratories and will be reported in a later paper in this series. In addition a long-term study of the closed-pan caramel in rats was undertaken (Evans, Butterworth, Gaunt & Grasso, 1977) to investigate the sequelae of the effects recorded here.

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LONG-TERM TOXICITY STUDY IN THE RAT ON A CARAMEL PRODUCED BY THE 'HALF OPEN-HALF CLOSED PAN' AMMONIA PROCESS

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Abstract—Caramel produced by a straight ammonia-catalysed 'half open-half closed pan' process was given at levels of 0 (control), 1, 3 or 6% in the diet to groups of 48 male and 48 female rats for 2 yr. In both sexes there was a reduced rate of body-weight gain at all dose levels. In the males, this was accompanied by a reduction in the cumulative food intake. There was evidence that dilution of the diet by the caramel also contributed to the reduction in body-weight gain. A reduction in numbers of white cells was associated in the early part of the study with a lymphocytopenia, which was present until wk 52 in the male rats fed the diet containing 6% caramel. The spleen weight was reduced in a dose-related manner. Changes in other organ weights were considered to be related to the difference in body weight between groups. A no-untoward-effect level was not established for the caramel used in this study, but there was no evidence of a carcinogenic effect.

INTRODUCTION

Caramels are a chemically ill-defined group of materials produced by the thermal modification of carbohydrates. They are defined only by their method of manufacture and intensity of colour and are chosen largely on the basis of requirements for colouring, flavouring or both.

Details of the chemistry of these compounds and the available toxicological data have been reviewed elsewhere (Gaunt, Lloyd, Grasso, Gangolli & Butterworth, 1977; Joint FAO/WHO Expert Committee on Food Additives, 1972a). A 90-day study conducted on two caramels produced by variations of the ammonia process (open pan and 'half open-half closed' or 'closed and open' pan) showed that rats fed on these caramels developed a dose-related enlargement of the caecum, a lymphocytopenia and a reduction in body weight and erythrocyte count (Gaunt *et al.* 1977). These changes were most pronounced among the males and in the animals fed diet containing the caramel produced by the 'half open-half closed pan' method.

The available information indicates that the 4-methylimidazole content of caramels may vary between 50 and 700 ppm, and in view of this and the data derived from animal feeding studies, the temporary Acceptable Daily Intake for caramels produced by the ammonia process was set at 0–100 mg/kg (Joint FAO/WHO Expert Committee on Food Additives, 1972a,b) on the basis of data on a product having a colour intensity of 20,000 European Brewing Convention (EBC) units and containing 200 ppm 4-methylimidazole. This view was endorsed by the Commission of the European Communities (1975) in a Report of the Scientific Committee for Food. This Committee requested the results of long-term and reproduction studies of samples of caramels prepared by the ammonia or ammonium sulphite process and containing a range of levels of 4-methylimidazole and recommended that the Acceptable Daily Intake set by the FAO/WHO Committee should be allowed until 31 December 1978.

The present paper describes the effects of long-term dietary administration to rats of caramel produced by the 'half open-half closed pan' ammonia process.

EXPERIMENTAL

Materials. The material used in this study was a straight ammonia-catalysed 'half open-half closed pan' caramel, referred to as 'Caramel' throughout this paper. It was supplied by Hay-Lambert Ltd., Uxbridge, Middx, and was similar to that used in the short-term study (Gaunt *et al.* 1977). Its specification was: Colour, 55,000 EBC units; pH (undiluted), 4·9; extract, 55·0 brewers' lbs/2 cwt; specific gravity (10% w/v solution at 15·5°C), 1·025; sulphated ash, 0·80% (w/w); iron, 49·7, lead, <0·01, copper, 2·3 and arsenic, 0·01 ppm (w/w); nitrogen, 5·98% (w/w); invert sugar, 12·01% (w/w); sucrose, 1·89% (w/w).

Animals and maintenance. Both sexes of rat used in this study were from a specified-pathogen-free colony of Wistar-derived animals (A. Tuck and Son, Rayleigh, Essex). They were housed in an animal room maintained at 20 ± 1°C with a relative humidity of 50–60%. The basal diet was Spillers' Laboratory Small Animal Diet which, together with water, was freely available.

Conduct of experiment. Groups of 48 male and 48 female rats were given diets containing 0 (control), 1, 3 or 6% Caramel for 2 yr. The animals were observed regularly, and any showing signs of ill health were isolated. They were returned to their group if their condition improved or, if not, were killed and subjected to post-mortem examination. The rats were weighed individually on the day before and 24 hr after

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the commencement of treatment, on days 3, 7, 14, 21 and 28 and then at monthly intervals until the end of the experiment. The quantity of food and water consumed by each cage of rats was measured over the 24 hr period preceding each day of weighing.

At wk 13, 26, 52 and 80, blood samples for haematological examination were collected from the tail veins of ten rats of each sex from the control group and those given diets containing 3 or 6% Caramel. After 104 wk, blood was collected from the aorta of all remaining animals during the post-mortem examination. Measurements were made of haemoglobin concentration and packed cell volume, together with total erythrocyte and leucocyte counts and differential leucocyte counts. Samples were collected also from ten rats of each sex given diets containing 0, 3 or 6% Caramel during wk 14 and from ten males from the same groups during wk 15 for additional total leucocyte counts. During wk 13 urine samples were collected from ten rats of each sex from the control group and the group given 6% of the test compound and examined for protein, glucose, bile salts, ketones and blood. At the same time the renal concentrating and diluting ability was assessed by measuring the volume and specific gravity of the urine produced during a 6-hr period of water deprivation and in the 2-hr period following a water load of 25 ml/kg. In addition the same measurements were made on the urine produced during a 4-hr period commencing after 16 hr without water. Numbers of cells were counted in the 2-hr urine samples.

Animals that died during the study were subjected to a post-mortem examination unless this was precluded by autolysis or cannibalism. All animals found *in extremis* during the study and those surviving to wk 104 were killed by an ip injection of barbiturate, bled from the aorta and examined for macroscopic abnormalities. The animals killed after wk 104 were deprived of food overnight before killing and the brain, heart, liver spleen, kidneys, stomach, small intestine, caecum (with and without its contents), adrenal glands, gonads, pituitary and thyroid were weighed. Samples of these tissues, together with salivary glands, thymus, various lymph nodes, pancreas, aorta, lungs, trachea, oesophagus, colon, rectum, skeletal muscle, spinal cord, uterus or prostate and any other tissues that appeared abnormal were preserved in buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for histological examination. In addition selected sections were stained by Perls' method. Wherever possible similar examinations were carried out on animals found *in extremis* between wk 101 and 104 and these data are included in the tables.

Table 1. *Cumulative mortality of rats fed diets containing 0–6% Caramel for 2 yr*

Wk on test	Total no. of deaths							
	Males given dietary levels (%) of				Females given dietary levels (%) of			
	0	1	3	6	0	1	3	6
16	0	0	0	0	0	1	0	1
48	2	1	0	0	1	2	2	2
64	3	2	1	4	2	3	4	3
80	7	6	5	7	6	6	4	9
88	12	11	13	9	8	15	7	9
96	16	13	21	15	9	19*	9	10
102	23	20	36**	21	19	25	14	20

The figures are the total number of animals dead or killed *in extremis* from groups of 48. Those marked with an asterisk differ significantly from the corresponding controls (by chi-square test):
*$P < 0.05$; **$P < 0.01$.

RESULTS

Apart from some discoloration of the fur by the Caramel, the appearance and behaviour of the rats given up to 6% Caramel did not differ in any way from those of the controls. Compared with the controls, there was no significant increase in the number of deaths among animals fed Caramel in the diet until wk 96 in the females and wk 102 in the males, when there were marginally more deaths in females fed 1% and in males fed 3% Caramel (Table 1). Body weights were significantly lower than those of the corresponding controls from wk 4 in males given 3 or 6% Caramel and from wk 13 in those given 1%. A lower body-weight gain among treated females after 1 yr was not statistically significant; the difference from controls was similar in females fed 1 or 3% Caramel but was more pronounced among those fed 6% (Table 2). The water consumption and the food intake (Table 2) showed no consistent differences between control and treated animals. In all groups the calculated intake of Caramel (Table 2) was higher over the first 3 months; for the remainder of the experiment it averaged, for males and females, respectively, 0·34 and 0·51/kg/day at the 1% level, 1·15 and 1·31 g/kg/day at 3%, and 2·25 and 3·1 g/kg/day at 6%. The calculated food conversion ratios (weight gain/weight of digestible food) for the treated animals showed no clear differences from the ratios for the corresponding controls.

No abnormal constituents were found in the urine and, apart from a marginally lower volume of urine in the females fed 6% Caramel during the 6-hr collection, no difference was found in the urinary concentration test or in cell excretion.

The results of the haematological examinations at wk 13, 52 and 104 are shown in Table 3. There were no statistically significant differences between control and treated animals in the erythrocyte counts, packed cell volumes or haemoglobin concentrations at these times or at wk 26 or 80, but significant reductions in the total leucocyte count were observed at wk 13 and 26 in the males fed 6% Caramel. Significant ($P < 0.001$) reductions in the numbers of these cells were also apparent in this group in the additional counts made at wk 14 and 15, and at wk 14 the numbers were also reduced significantly in the females given 6% Caramel ($P < 0.001$) and in the males given 3% ($P < 0.05$). The only other significant differences in the haematological data between control and treated animals were a raised leucocyte count in females at wk 80 and a reduced count in males at 2 yr, both again in the group fed 6% Caramel. The reduced count among males at 2 yr was associated

with a reduced number of neutrophils, but in the main the leucopenia, which was most marked in males, was associated with a lowering of the lymphocyte to neutrophil ratio. The total percentages of monocytes, eosinophils and basophils in the differential counts were less than 3·5% and the actual numbers of these have therefore not been tabulated. When absolute figures were calculated, the general trend of higher neutrophil and lower lymphocyte numbers was repeated, although among treated females, the lymphocyte count was significantly different from that of the corresponding controls only at the terminal examination in rats fed 6% Caramel. In the males, the absolute lymphocyte numbers were lower in the earlier part of the study than the corresponding control values, the differences being statistically significant at wk 13 in animals fed 3 or 6% Caramel, and in animals fed 6% Caramel also at wk 26 and 52. The neutrophil numbers were significantly higher in all the treated males in which values were recorded, other than at wk 52 in males fed 3% Caramel.

In both sexes, organ weights generally reflected body weights. Spleen weights were lower in the treated rats than in the controls, the differences, which were dose-related, being statistically significant in the males at all treatment levels and in the females fed 3 or 6% Caramel. Heart and liver weights were significantly lower in treated males than in the controls, the former in the groups fed 3 or 6%, the latter in all the treated groups. Stomach weights in treated males were also below those of the controls, the differences being statistically significant in those fed 1 or 6% Caramel, and in the latter group the small-intestine weight was also lower. Kidney weights exceeded the control value in treated females with a statistically significant difference at the 3% level. These differences were largely eliminated when the weights were expressed relative to body weight (Table 4). However, the relative weights of the brain and of the full caecum were higher than the control values in males fed the two higher dietary levels, while in females, the relative weights of the full caecum in those fed 1 or 6% Caramel and of the thyroid and kidney in those fed 6 and 3%, respectively, were significantly higher than the control values.

The histopathological lesions encountered during this study are shown in Tables 5 and 6 and, in general, were found with similar frequency in control and treated animals. In females fed 3% and males fed 1

Table 2. *Mean body weights and food and colouring intakes of rats fed diets containing 0–6% Caramel for 2 yr*

Sex and dietary level (%)	Value at day		Value at wk									
	0†	3	2	4	9	13	26	39	53	65	78	101
						Body weight (g)						
Male												
0	67	85	172	260	387	445	524	589	655	698	717	701
1	68	86	170	256	374	412***	482***	549**	596***	626***	643***	625***
3	68	85	169	250*	357***	405***	485***	550**	594***	625***	622***	579***
6	66	85	167	245***	356***	405***	466***	523***	565***	590***	592***	572***
Female												
0	68	83	143	179	230	252	289	321	364	397	430	456
1	67	83	141	182	235	253	292	323	361	389	417	418
3	67	83	140	179	236	254	290	324	359	391	417	432
6	68	83	142	182	231	251	278	308	337	369	392	416
						Food intake (g/rat/day)						
Male												
0	11·0	17·5	20·1	25·7	23·1	21·7	22·8	21·7	22·6	19·6	22·5	22·8
1	11·1	17·6	19·3	24·3	16·4	22·6	20·4	22·8	20·5	19·9	21·6	19·8
3	20·4	17·1	19·5	23·1	21·2	21·2	19·9	21·7	21·8	18·9	21·4	19·2
6	11·4	16·1	18·5	23·5	19·5	22·4	19·7	21·9	22·1	19·5	22·2	21·6
Female												
0	10·6	15·8	15·9	17·5	17·1	16·8	17·1	17·6	19·0	15·1	18·0	21·6
1	10·6	15·8	15·9	18·8	16·7	17·2	17·5	20·8	18·0	16·4	19·6	19·5
3	13·2	14·9	16·2	17·9	18·3	18·6	14·9	17·9	17·6	16·4	19·6	20·5
6	9·7	15·7	16·6	20·2	18·6	19·3	16·4	18·3	17·1	17·3	20·6	18·2
						Colouring intake (g/kg/day)						
Male												
1	1·62	2·03	1·14	0·95	0·64	0·55	0·42	0·40	0·35	0·32	0·37	0·32
3	8·97	6·87	3·50	2·77	1·78	1·57	1·32	1·18	1·08	0·91	1·03	1·00
6	10·01	11·91	6·65	5·64	3·28	3·32	3·54	2·51	2·34	1·98	2·25	2·27
Female												
1	1·57	1·90	1·12	1·03	0·71	0·68	0·60	0·64	0·50	0·42	0·47	0·47
3	5·87	5·43	3·47	2·98	2·33	2·19	1·54	1·66	1·47	1·25	1·37	1·42
6	8·55	11·20	7·00	6·70	4·80	4·60	3·50	3·55	3·05	2·80	3·15	2·61

†First day of feeding.
The figures for food intake are the means for all surviving rats. Those for colouring intake are calculated from data on food intake and body weight.

or 3% Caramel, there were statistically significant reductions in the incidence of interstitial pneumonitis in females fed 3% caramel and of focal centrilobular vacuolation of the liver in males fed 1 or 3%. In females fed 3 or 6% Caramel the incidence of bile-duct hyperplasia was higher than in the controls, although the increase was only statistically significant in the 3% group.

The tumour found most frequently in either sex was an adenoma of the pituitary. In addition the females had many benign mammary tumours. However, in both cases there was no statistically significant difference between the treated groups and the controls. The number of malignant tumours was low and included three carcinomas of the mammary gland in control females as well as one, two and one in the groups given 1, 3 and 6% Caramel, respectively, and two carcinomas of the thyroid in females fed 1% Caramel in the diet. Adenocarcinoma of the pancreas was found in two male and two female controls and in two males fed 1% Caramel as well as in one male and one female fed 6% Caramel. An ovarian carcinoma was found in one female control animal, in two animals fed the diet containing 3% Caramel and in three fed 6% Caramel. Lymphosarcoma was seen in a single male fed a dietary level of 1% Caramel and in one female from each of the groups fed diet containing 0, 1 and 6%. A uterine leiomyosarcoma was seen in a control animal and in an animal fed 6% Caramel. Carcinomas of the adrenal cortex occurred in two males fed 3% Caramel and in one female from each of the groups fed 0, 1 and 3%. Also isolated instances of other malignant tumours were seen. Among males, there was a squamous-cell carcinoma, a basal-cell carcinoma and a subcutaneous reticulum-cell sarcoma in the control group and a meningioma and a carcinoma of the salivary gland were found in treated rats fed 1 and 3% Caramel respectively. There was also a haemangiosarcoma in the uterus of a single female fed diet containing 1% Caramel and a subcutaneous fibrosarcoma in one male control and one given 1% Caramel.

Table 3. *Haematological findings in rats fed diets containing 0,3 or 6% Caramel for 13, 52 or 104 wk*

Sex and dietary level (%)	No. of rats	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Total WBC (10^3/mm^3)	Differential WBC count (%)				Calculated numbers of WBC (10^3/mm^3)	
						L	M	E	N	L	N
					Wk 13						
Male											
0	10	16·0	49	7·50	11·2	87	1	1	11	8·9	1·2
3	10	16·5	48	6·78	10·4	70**	1	2	26**	7·3**	2·7**
6	10	16·7	49	7·14	8·4***	60***	2	0	38***	4·9***	3·1***
Female											
0	10	15·4	46	7·32	7·9	82	1	0	16	6·5	1·3
3	10	15·7	47	7·47	8·5	70*	1	2	27	5·9	2·3*
6	10	15·3	46	7·51	8·5	70*	0	0	25	5·8	2·4*
					Wk 52						
Male											
0	10	16·4	49	7·81	13·0	80	0	0	20	10·7	2·5
3	10	16·6	50	7·64	12·3	70*	0	0	30*	8·6	3·0
6	10	16·1	47	7·63	11·1	62***	1	0	37***	6·8***	3·8*
Female											
0	10	16·4	48	7·65	7·8	69	1	0	31	5·3	2·5
3	10	17·4	48	7·22	8·2	69	0	0	34	5·3	2·8
6	10	16·6	47	6·93	7·3	62	1	0	38	4·5	2·7
					Wk 104						
Male											
0	27	12·8	41	6·50	5·6	—	—	—	—	2·7	2·5
1	30	12·7	40	6·94	5·2	—	—	—	—	—	—
3	22	13·6	40	7·03	4·8	—	—	—	—	—	—
6	31	12·8	40	6·82	4·3*	—	—	—	—	2·5	1·6**
Females											
0	35	13·3	40	6·24	3·7	—	—	—	—	2·2	1·4
1	35	13·7	42	6·72	4·3	—	—	—	—	—	—
3	36	13·6	41	6·62	3·7	—	—	—	—	—	—
6	33	13·6	42	6·84	3·4	—	—	—	—	1·8**	1·6

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

These figures are the means for the numbers of rats shown and those marked with an asterisk differ significantly (Student's t test) from the appropriate control value: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.
Basophils did not constitute more than 0·5% of the white blood cells in any group.

Table 4. *Organ weights and relative organ weights of rats fed diets containing 0–6% Caramel for 2 yr*

Sex and dietary level (%)	No. of rats examined	Relative organ weight (g/100 g body weight)													Terminal body weight (g)
									Caecum						
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Full	Empty	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
Male															
0	30	0·35	0·28	2·66	0·22	0·80	0·47	1·60	0·59	0·20	11·5	0·55	1·99	4·42	620
1	32	0·38	0·28	2·59	0·20	0·74	0·46	1·65	0·71	0·22	10·6	0·62	1·96	4·60	568
3	22	0·42**	0·30	2·86	0·19	0·91	0·52	1·73	0·77*	0·24	12·9	0·70	1·98	4·49	507***
6	33	0·42***	0·30	2·95	0·18	0·84	0·49	1·71	0·74*	0·22	13·1	0·65**	2·42	4·94	518***
Female															
0	38	0·50	0·30	2·71	0·24	0·58	0·45	1·96	0·73	0·25	17·3	20·4	3·88	4·88	407
1	30	0·50	0·31	2·73	0·23	0·63	0·45	1·96	0·90*	0·25	16·8	20·9	4·52	4·89	393
3	36	0·52	0·32	3·07	0·22	0·70*	0·52	2·09	0·78	0·27	16·6	20·5	4·67	5·29	391
6	36	0·54	0·31	2·84	0·20	0·66	0·47	2·04	0·87**	0·27	17·2	21·6	4·45	5·87*	379

†Values for these organs are expressed in mg/100 g body weight.
‡Values for female gonads are expressed in mg/100 g body weight.
Values are the means for the numbers of rats shown and those marked with asterisks differ significantly (Student's *t* test) from the corresponding control figure: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 5. *Histopathological findings (excluding tumours) in rats fed diets containing 0–6% Caramel for 2 yr*

Tissue and finding	No. of rats examined...	Males 0 (48)	Males 1 (46)	Males 3 (39)	Males 6 (45)	Females 0 (47)	Females 1 (45)	Females 3 (46)	Females 6 (46)
		No. of animals affected among							
		Males given dietary level (%) of				Females given dietary level (%) of			
Heart									
Fibrosis		15	19	7	9	4	10	2	10
Myocarditis		1	0	0	0	0	0	0	0
Aorta									
Calcification		5	4	5	5	0	0	0	0
Lung									
Interstitial pneumonitis		16	17	7	10	17	13	8*	12
Calcification of alveolar wall		0	0	0	3	5	0	0	0
Periarteritis		0	0	0	0	1	1	0	0
Kidney									
Glomerulonephrosis		38	43	34	35	47	40	36	32
Cystic (large)		1	0	1	0	0	0	0	0
Hydronephrosis		0	0	0	0	0	1	0	0
Pyelonephritis		0	0	0	0	0	1	0	0
Heavy focal lymphocytic infiltration		0	0	1	0	0	0	0	0
Liver									
Focal neutrophil infiltration		1	0	0	0	0	0	0	0
Focal centrilobular fatty change		21	10*	12	4**	7	7	8	13
Generalized fatty change		2	1	2	0	3	2	1	0
Bile-duct hyperplasia		7	8	1	4	6	5	15*	12
Sinusoidal dilation		2	3	1	1	1	0	0	1
Small nodule		3	0	2	3	0	0	3	3
Periarteritis		0	0	0	0	1	0	0	1
Spleen									
Arteritis		3	0	0	0	1	0	0	0
Nodular areas with normal histology		1	0	0	0	0	0	0	0
Stomach									
Arteritis		1	2	0	0	0	0	0	0
Calcification		3	3	3	5	0	0	0	0
Gastritis		1	1	0	0	2	1	2	4
Oedema (severe)		0	0	0	0	1	0	0	0
Large intestine									
Inflammation		1	1	1	0	2	0	1	2
Brain									
Vacuolation of white matter		2	0	0	0	2	0	0	1
Harderian gland									
Fibrosis		0	0	0	0	0	0	1	0
Pancreas									
Arteritis		1	4	4	2	2	1	3	5
Large islet		0	0	0	0	0	1	0	1
Fibrosis with ductal hyperplasia		0	0	1	1	0	2	0	2
Focal hyperplasia		0	2	0	2	0	0	0	0
Thyroid									
Colloid cyst		0	0	0	0	1	0	0	0
Hypertrophy		0	1	0	0	1	1	0	0
Clear-cell hyperplasia		0	0	0	3	0	0	0	1
Arteritis		0	0	0	1	0	0	0	0
Hyperplastic nodule		0	0	0	1	0	0	0	0
Adrenal gland									
Vacuolation in cortex		9	12	4	3	3	1	4	6
Pigment		0	0	0	0	1	1	0	0
Cortical hyperplasia		1	2	2	0	2	5	0	1
Adrenal									
Arteritis		0	0	0	0	0	1	0	0
Calcification		0	0	0	1	0	0	0	0
Pituitary									
Hyperplastic nodule		1	1	1	1	0	0	0	0
Salivary gland									
Inflammation		3	1	0	2	0	1	0	1
Bladder									
Cystitis		1	0	1	0	0	0	1	0

Table 5. *Continued*

Tissue and finding	No. of rats examined...	Males 0	Males 1	Males 3	Males 6	Females 0	Females 1	Females 3	Females 6
		No. of animals affected among							
		Males given dietary level (%) of				Females given dietary level (%) of			
		0	1	3	6	0	1	3	6
		48	46	39	45	47	45	46	46
Skin and subcutaneous tissue									
Chronic inflammatory changes		1	2	1	1	0	0	1	2
Testes									
Periarteritis		12	17	18	13	—	—	—	—
Atrophy		11	11	5	3	—	—	—	—
Oedema		0	1	1	0	—	—	—	—
Ovaries									
Cysts		—	—	—	—	3	1	1	1
Pigmentation		—	—	—	—	0	1	0	0
Atrophy		—	—	—	—	3	1	1	1
Seminal vesicles									
Inflammation		0	0	0	2	—	—	—	—
Lymph nodes									
Pigmentation (Perls' stain): +		3	4	3	4	16	11	14	13
++		2	1	3	1	3	9	3	10
Uterus									
Endometritis with or without cyst formation		—	—	—	—	2	3	3	5
Pyometra		—	—	—	—	6	0	0	1

The figures represent the incidence of findings among the numbers of rats shown and those marked with asterisks differ significantly (chi-square test) from those of the controls: *$P < 0.05$; **$P < 0.01$.

DISCUSSION

In a previous study (Gaunt *et al.* 1977) a dose-related reduction in weight gain was reported in both male and female rats fed caramels produced by two (open-pan and 'half open-half closed' pan) variations of the ammonia process. When the dilution effect of caramel in the diet was taken into consideration, the food conversion ratio was similar to that of the controls at the 4% dietary level for the latter type of caramel but was reduced at 8 and 16%. It was suggested, therefore, that some factor other than dilution of the diet was affecting the weight gain at the higher levels. In the present study, there was a reduction in weight gain in both males and females in the treated groups, but the reduction was statistically significant only in the males. When food conversion ratios were calculated, no clear difference emerged between the treated groups and their corresponding controls, other than a possible slight reduction in food intake in males fed 6% Caramel. These findings suggest that the reduction in body-weight gain among the rats fed Caramel at levels up to 6% of the diet was caused by a dilution effect of the test material in the diet, with the effect in males being exacerbated by a reduction in food intake among the treated animals. In this evaluation a digestibility coefficient of 75% was used for the basal diet (Spillers' data sheet) and 50% for caramel (Joint FAO/WHO Expert Committee on Food Additives, 1972a).

As in the previous study (Gaunt *et al.* 1977), a reduction in the leucocyte count was seen in both males and females during the early part of this 2-yr study. This reduction remained evident in males until wk 52. In general the reduction was accompanied by a reduced lymphocyte count and an increase in the number of neutrophils. The reason for this effect is not clear. It appears not to be related to a reduced food intake, since food restriction to some 10% of normal is required to produce a lymphocytopenia of this magnitude (Shukers & Day, 1943), a degree of restriction much more severe than that seen in the present experiment. In the absence of other data, it must be assumed, at present, that the reduction in the number of lymphocytes was associated with the Caramel treatment. The reason for the apparent recovery towards the end of the study may be related to the normal ageing changes in the rat. The reduced number of neutrophils in the male animals at 2 yr does not follow any definite pattern and appears to be an isolated finding unrelated to the Caramel administration.

In all the groups, including the controls, the total leucocyte counts and the calculated totals for the different types of white cells were lower at 2 yr than in the earlier examinations. This difference can be attributed to the different technique used for obtaining the terminal blood samples (Gaunt *et al.* 1977).

Reduced absolute organ weights associated with normal or slightly raised relative weights have been reported for animals with low body-weight gains resulting from a reduced intake of normal diet (Schwartz, Tornaben & Boxill, 1973) or from consumption of diets containing indigestible cellulose (Feron, de Groot, Spanjers & Til, 1973), and an increase in the relative brain weight in such animals has been described (Weil, 1970). Therefore, reductions

in the weights of the heart, liver and empty caecum accompanied by a brain weight similar to that in the controls were not unexpected in animals which had a reduced food intake due, presumably, to an unpalatable diet. These weight changes cannot be taken, therefore, as evidence of a toxic effect.

With the spleen, the situation is more complex. In this particular study both the absolute and relative spleen weights were reduced, although the reductions in the relative weights were not statistically significant. If the reduced spleen weights had resulted solely from inanition, the relative weights would have been likely to be similar to or marginally higher than those of the corresponding controls (Schwartz *et al.* 1973). It must be presumed, therefore, that the reduction in spleen weight was due in part to the feeding of the Caramel. A large proportion of circulating lymphocytes (Humphrey & White, 1970) and of splenic cells (Jandl, Files, Barnett & MacDonald, 1965) are small lymphocytes. Thus it is possible that the reductions in the numbers of circulating lymphocytes during the experiment and in the terminal spleen

Table 6. *Incidence of tumours in rats fed diets containing 0–6% Caramel for 2 yr*

		No. of animals with tumours among							
		Males given dietary level (%) of				Females given dietary level (%) of			
	No. of rats	0	1	3	6	0	1	3	6
Organ/Tumour	examined...	48	46	39	45	47	45	46	46
Liver									
Adenoma		0	0	0	0	1	0	0	0
Lung									
Squamous-cell carcinoma		1	0	0	0	0	0	0	0
Pancreas									
Insuloma		1	1	1	0	0	0	1	0
Adenocarcinoma		2	2	0	1	2	0	0	1
Pituitary									
Adenoma		4	2	8	3	14	15	18	14
Adrenal									
Medullary adenoma		1	0	0	0	0	0	0	0
Carcinoma		0	0	2	0	1	1	1	0
Cortical adenoma		0	0	0	0	0	0	1	0
Thyroid									
Adenoma		0	0	0	3	2	1	0	2
Carcinoma		1	0	0	0	0	2	0	0
Skin and subcutaneous tissue									
Reticulum-cell sarcoma		1	0	0	0	0	0	0	0
Fibroma		2	3	1	2	0	0	0	0
Fibrosarcoma		1	0	1	0	0	0	0	0
Squamous-cell carcinoma		1	0	0	0	0	0	0	0
Basal-cell carcinoma		1	0	0	0	0	0	0	0
Mammary gland									
Carcinoma		0	0	0	0	3	1	2	1
Adenoma or fibroadenoma		0	0	0	0	12	12	12	9
Fibroma		0	0	0	0	1	1	0	1
Ovary									
Carcinoma		0	0	0	0	1	0	2	3
Uterus									
Cystadenoma		0	0	0	0	0	1	0	0
Leiomyosarcoma		0	0	0	0	1	0	0	1
Haemangiosarcoma		0	0	0	0	0	1	0	0
Fibroadenoma		0	0	0	0	1	1	0	0
Testis									
Interstitial-cell tumour		5	3	2	1	0	0	0	0
Kidney									
Hamartoma		0	0	1	0	0	0	0	0
Peritoneum									
Lipoma		1	0	0	0	0	0	1	0
Salivary gland									
Carcinoma		0	0	1	0	0	0	0	0
Adenoma		0	0	0	0	0	0	0	1
Brain									
Meningioma		0	1	0	0	0	0	0	0
Lymph node									
Lymphosarcoma		0	1	0	0	1	1	0	1

The figures represent the incidence of the finding among the numbers of rats shown.
No incidence in the treated groups differed significantly ($P < 0.05$ by chi-square test) from that in the controls.

weights of treated animals were both manifestations of the same underlying process.

The isolated increase in kidney weight in females fed 3% Caramel was not associated with any histopathological lesion and was probably unrelated to treatment since there was no statistically significant increase at the 6% level or in the males.

The histological lesions observed in this study occurred with similar frequency in control and treated animals. The slight increase in bile-duct hyperplasia seen in females fed 3% Caramel in the diet was not considered to be of any biological significance as this lesion is often present in old rats.

Most of the tumours in the present experiment were found in controls alone or with similar frequency in control and treated animals. Generalized lymphosarcomas have been described previously in control rats in our laboratory (Gaunt, Butterworth, Hardy & Gangolli, 1975). Similarly, mammary adenocarcinomas, carcinomas of the thyroid and ovarian carcinomas have all been described in untreated animals (Snell, 1965). Four other isolated malignant tumours that occurred in treated but not in control animals have also been reported previously in untreated animals. Although uncommon, carcinomas of the adrenal cortex have been described (Russfield, 1967), as have carcinomas of the salivary gland and meningiomas affecting the brain (Snell, 1965). Malignant tumours of vascular tissue, although not common in the genital tract, have been reported. Thompson, Huseby, Fox, Davis & Hunt (1961) recorded a haemangioendothelioma of the oviduct in a Sprague–Dawley rat, while Snell (1965) states that although the majority of uterine tumours in old Wistar rats are adenomatous polyps a few angiosarcomas also occur.

It is concluded that this study failed to demonstrate any carcinogenic effect on the part of Caramel fed at levels up to 6%. However, like the earlier, short-term, experiment (Gaunt *et al.* 1977), it demonstrated a lymphocytopenia with dietary levels of 3% and above and a reduced splenic weight probably related to the lymphocytopenia. In addition the splenic weight differed from the controls in animals on the low level of treatment (1%) suggesting that the same syndrome was developing at this dose. Because of these two probably inter-related observations, it was not possible to establish a no-untoward-effect level. The final evaluation of these findings must depend on the outcome of investigations of the mechanism underlying the observed effects.

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LONG-TERM EFFECTS OF CALCIUM CARRAGEENAN IN RATS—I. EFFECTS ON REPRODUCTION

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Abstract—Long-term multigeneration effects of the dietary intake of calcium carrageenan were measured in a three-generation reproduction and teratology study in Osborne–Mendel rats. Dietary levels of 0·5, 1·0, 2·5 or 5·0% were ingested throughout the study. Carrageenan ingestion caused dose-related and significant decreases in the weights of offspring at weaning, but no effects were detected in respect of fertility, average litter size, average number of liveborn animals, viability or survival of offspring.

INTRODUCTION

Carrageenan is the generic name for a group of calcium, sodium, potassium and other salts of sulphated polysaccharides that are present in many species of red algae and are used extensively in food. The three main types of carrageenan, iota, kappa and lambda, are differentiated on the basis of type, location and number of sulphate groups. Commercial carrageenans for use in foods are of high molecular weight and are generally mixtures of κ- and λ-carrageenan. Degraded carrageenan is of low molecular weight and usually refers to ι-carrageenan. In some reports, authors have not specified the type of carrageenan used.

Carrageenan of all types was included by the Food and Drug Administration (FDA) in the first list of GRAS (Generally Recognized As Safe) compounds (*Federal Register* 1959, **24**, 9368). Two years later, the compound was listed as a regulated food additive (*ibid* 1961, **26**, 9411). This listing was followed by the publication of an acceptable daily intake (ADI) of 50 mg/kg body weight, or 3·5 g/day for an average 70-kg man, by the Joint FAO/WHO Expert Committee on Food Additives (1970).

Questions arose concerning the safety of continued use of carrageenan in food, since a series of studies were reported in which rabbits, guinea-pigs and rats developed ulcerations of the caecum and proximal colon after being given drinking-water containing degraded carrageenan (Marcus & Watt, 1969; Watt & Marcus, 1969 & 1970a,b). The results of these studies were contested by many investigators, including Bonfils (1970), Maillet, Bonfils & Lister (1970) and Sharratt, Grasso, Carpanini & Gangolli (1971). To guard against the introduction of degraded carrageenan, the FDA (*Federal Register* 1972, **37**, 15434) stated that only carrageenan with a molecular weight exceeding 100,000 could be used in food.

Despite the long and widespread usage of carrageenan in food, relatively few long-term feeding studies have been performed. Nilson & Wagner (1959) reported that rats had a slower growth rate and some hepatic lesions after ingestion of a diet containing 25% of the high-molecular-weight compound for 24 months. Hawkins & Yaphe (1965) noted a dose-related decrease in the growth rate of rats fed 2–20% high-molecular-weight carrageenan in the diet for approximately 21 wk. No adverse dose-related effects were noted in pigs that had ingested the high-molecular-weight compound at levels up to 500 mg/kg/day for 12 wk (Poulsen, 1973), nor in infant baboons given κ,λ-carrageenan in infant formula at dose levels up to 1500 mg/litre for 16 wk (FDA files, unpublished data submitted by the Infant Formula Council). Monkeys given high-molecular-weight carrageenan in the drinking-water (average daily intake 1·3 g/kg/day) for 11 wk and then given escalating daily doses of 50–1250 mg/kg by stomach tube for up to 12 wk all remained in good condition (Benitz, Golberg & Coulston, 1973).

Pittman, Golberg & Coulston (1976) fed rats a diet containing carrageenan of high molecular weight for 13 wk and found little or no absorption of the carrageenan, as indicated by the absence of the compound from the livers of the animals killed at the end of the feeding period.

In our own laboratory, a pilot study was conducted to compare rats fed 25% dietary calcium κ,λ-carrageenan with rats fed a control diet. The animals fed carrageenan lost weight rapidly, presented an unthrifty appearance and had pronounced diarrhoea. It became obvious that this level of carrageenan could not be tolerated by the animals for a long-term study. These results, together with the lack of information on the long-term effects of carrageenan ingestion combined with the additional stress of pregnancy and other results obtained in foetotoxicity studies (Food and Drug Research Laboratories, Inc., 1973a,b), prompted this combined multigeneration reproduction and teratology study.

EXPERIMENTAL

Materials and animals. Calcium carrageenan, Gelcarin-HMR (Lot No. 102012, Marine Colloids, Inc.,

Rockland, Maine), was mixed with Purina Rat Chow (Ralston-Purina Co., Inc., St. Louis, Mo.) at levels of 0·5, 1·0, 2·5 or 5·0% (5000–50,000 ppm). The major components of this κ,λ-carrageenan were: alcohol precipitables, 97·57%; sulphate ester, 24·43%; free sulphate, 2·44%; 3,6-anhydro-*d*-galactose, 22·98%; water, 9·41%; potassium, 5·95%; calcium, 2·51%; sodium, 1·24%; chloride, 1·02%; nitrogen, 0·21%; magnesium, 0·04%. The viscosity of a 1·5% solution was 195·0 cP at 75°C. Osborne–Mendel rats were obtained from the FDA breeding colony. The carrageenan-containing diets were fed to the experimental animals throughout the study, and unadulterated rat chow was fed to the control animals.

Experimental procedure. The protocol for the three-generation study is diagrammed in Fig. 1. Forty males and 40 females were used at each dose level. After weaning, all animals were fed carrageenan in their diets for 12 wk before mating. Animals of both F1A and F1B litters were sexed and weighed at birth and weighed also on days 4, 7, 14 and 21. A careful check for abnormalities was made of all rats until weaning at 21 days, when F1A animals were autopsied and then discarded. Randomly selected F1B and F2B animals were mated to produce the F2 and F3 generations, respectively. F2A, F2B, F3A and F3B animals were weaned and autopsied in the same manner as F1A animals, except that randomly selected rats from F3A were used in a chronic feeding study, which will be reported in a separate paper. On day 20 of pregnancy, the F2C and F3C litters were exposed by caesarean section. The results of the study on foetal development are reported separately (Collins, Black & Prew, 1977).

Randomly selected F0, F1B and F2B parental animals were maintained on the same dietary level of carrageenan for 9 months and submitted for pathological examination. Preliminary findings reported at a meeting between industrial representatives and the FDA showed that dose-related liver lesions occurred at the two highest dose levels (*Food Chemical News*, 1974). Representative weanlings from F2A, F2B, F3A and F3B were also examined for pathology. Complete pathological findings of the liver lesions that appeared in the adults of the three generations and in the third-generation weanlings will be described in a separate paper.

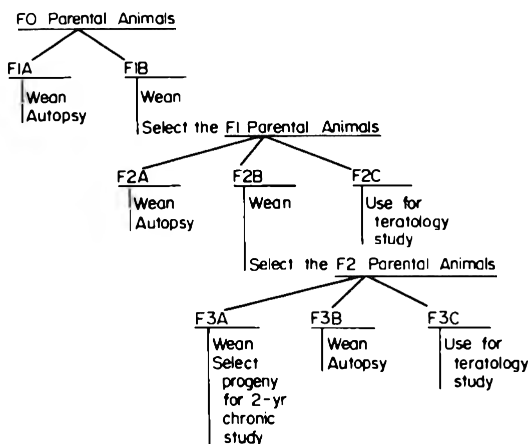

Fig. 1. Protocol for three-generation reproduction and teratology study of calcium carrageenan in rats.

All adult rats were weighed weekly and food consumption was measured at the same time. All litters of more than ten offspring were reduced to ten by a randomized selection on day 4. When killed, all adult and weanling rats were autopsied.

The rats were housed individually in screen-bottom cages with free access to food and water.

Statistical analyses. The fertility indexes were analysed for statistical significance by the two-tailed chi-square test. Data for viability, survival and weaning indexes were transformed by the Freeman–Tukey arc-sine transformation for binomial proportions (Mosteller & Youtz, 1961) followed by analysis of variance and a least significant difference (LSD) test. The average litter size, number of liveborn and number of survivors were analysed by analysis of variance and an LSD test. Weights were also analysed by an analysis of variance and an LSD test. Analyses of weight gain and food consumption in dams for 12 wk were based on means adjusted for initial weight by analysis of covariance. The adjusted means were analysed by an LSD test.

RESULTS

Carrageenan consumption had no effect on mortality, but there was a noticeable deterioration in the physical state of rats that received the 5·0% level. The rats that received the high dose levels were smaller and thinner than the controls, and had rough hair coats. Diarrhoea was marked in animals fed the two highest dose levels. The presence of carrageenan in the feed did not seem to detract from the palatability of the diet, as all the animals ate it, and consumption was increased, in some cases significantly, at the 2·5 and 5·0% levels (Table 1). Despite the consumption of the same or a greater quantity of food, the cumulative weight gain of the males at wk 12 after weaning was significantly reduced at the 5·0% level in F0, at the three highest dose levels in F1B and at all four dose levels in F2B (Table 1). The cumulative weight gain of the females during the same premating period was unaffected except for a significant decrease in F2B at the 5·0% level. The average daily carrageenan consumption for adult males was 290, 592, 1555 and 3212 mg/kg for the 0·5, 1·0, 2·5 and 5·0% levels, respectively. For the same dietary levels, the average daily carrageenan consumption in adult females was 350, 711, 1832 and 3805 mg/kg.

The fertility index, the (number of litters cast/number of females exposed to mating) × 100, reflects the female's ability to produce young. As seen in Table 2, no dose-related difference occurred at any dose level, but within each generation the fertility was decreased between the first and second litters for each dose level except at 1·0% in the first generation. Neither the average litter size (Table 3) nor the viability of the animals in the litter (Table 4) was affected by ingestion of the compound. The viability index, the (number of liveborn/total number born) × 100, reflects the loss of young through stillbirths; there was no indication that the compound had any visible effect on the production of live young. The average number of liveborn animals surviving to day 4 (Table

Table 1. *Mean weight gain and food consumption in rats fed calcium carrageenan for a 12-wk premating period*

Generation/ litter	Dietary level (%)	Weight gain (g)†		Food consumption (g)†	
		Male	Female	Male	Female
F0	0	361·2	194·4	1738·0	1306·6
	0·5	350·4	194·8	1727·6	1289·1
	1·0	348·8	194·6	1730·7	1332·6
	2·5	351·9	192·5	1814·2***	1379·0***
	5·0	327·0****	187·7	1745·5	1343·9
F1B	0	346·6	188·0	1770·0	1333·6
	0·5	348·0	189·7	1776·3	1284·1
	1·0	329·3*	186·6	1728·4	1278·3
	2·5	329·4*	182·9	1759·8	1329·0
	5·0	307·8****	183·4	1692·6	1310·2
F2B	0	367·9	190·4	1842·5	1397·4
	0·5	347·4**	186·1	1794·0	1380·2
	1·0	342·6***	192·4	1843·5	1434·8
	2·5	348·5*	195·1	1941·2***	1507·0***
	5·0	309·5****	174·2***	1846·2	1462·6*

†Statistical analyses are based on means adjusted to the initial weights of 39–41 animals. Values marked with asterisks differ significantly from the control value: *$P < 0·05$; **$P < 0·02$; ***$P < 0·01$; ****$P < 0·001$.

Table 2. *Fertility indexes of rats fed calcium carrageenan for three generations*

Dietary level (%)	Generation/litter...	Fertility index*					
		F1A	F1B	F2A	F2B	F3A	F3B
0		97·5 (40)	95·0 (40)	97·4 (39)	92·1 (38)	94·4 (39)	94·9 (39)
0·5		95·0 (40)	92·5 (40)	97·5 (40)	95·0 (40)	97·4 (39)	94·7 (38)
1·0		95·0 (40)	97·4 (39)	94·9 (39)	92·1 (38)	97·5 (40)	92·5 (40)
2·5		100·0 (40)	97·4 (39)	100·0 (40)	97·5 (40)	97·5 (40)	90·0 (40)
5·0		95·0 (40)	92·5 (40)	90·0 (40)	89·7 (39)	100·0 (41)	95·0 (40)

*(No. of pregnant females/no. of females exposed to mating) × 100. Figures in parentheses are the numbers of females exposed to mating in each group.

Table 3. *Average litter size of rats fed calcium carrageenan for three generations*

Dietary level (%)	Litter size in generation/litter...											
	F1A		F1B		F2A		F2B		F3A		F3B	
	No.*	Mean†	No.	Mean	No.	Mean	No.	Mean	No.	Mean	No.	Mean
0	428	11·3	458	12·1	400	10·5	406	11·6	433	11·4	452	12·2
0·5	400	10·5	438	11·8	425	10·9	427	11·2	432	11·4	432	12·0
1·0	442	11·6	472	12·4	368	9·9	348	9·9	429	11·0	410	11·1
2·5	438	11·0	476	12·5	448	11·2	468	12·0	454	11·6	430	11·9
5·0	419	11·0	458	12·4	389	10·8	406	11·6	460	11·2	458	12·1

*Total no. of progeny.
†Average litter size/pregnant female.

5) indicated that carrageenan had no significant effect at any dose level. The values obtained for the 4-day survival index, the (number of progeny alive at day 4/number born alive) × 100, indicate that carrageenan had no appreciable postnatal effects on the survival of the young. The only statistically significant decrease occurred at the lowest dose level and was not considered to be biologically significant.

Carrageenan appeared to have no effect on the viability of the young from birth to weaning at day 21 (Table 6); however, the weights of the animals at birth appeared to be decreased in the experimental animals (Table 7), especially at the two highest dose levels, although the decreases were significant in only four litters. Decreases in weanling weights were dose-related and statistically significant (Table 7).

DISCUSSION

It appears from this multigeneration study that calcium carrageenan of the kappa lambda type has no

Table 4. *Viability indexes of rats fed calcium carrageenan for three generations*

Dietary level (%)	Data on liveborn progeny in generation/litter . . .																	
	F1A			F1B			F2A			F2B			F3A			F3B		
	No.*	Mean†	VI	No.	Mean	VI	No.	Mean	VI	No.	Mean	VI	No.	Mean	VI	No.	Mean	VI
0	418	11·0	97·7	445	11·7	97·2	395	10·4	98·8	400	11·4	98·5	424	11·2	97·9	432	11·7	95·6
0·5	392	10·3	98·0	425	11·5	97·0	418	10·7	98·3	423	11·1	99·1	426	11·2	98·6	429	11·9	99·3
1·0	433	11·4	98·0	466	12·3	98·7	359	9·7	97·6	338	9·7	97·1	420	10·8	97·9	399	10·8	97·3
2·5	433	10·8	98·9	468	12·3	98·3	442	11·0	98·7	457	11·7	97·6	449	11·5	98·9	412	11·4	95·8
5·0	410	10·8	97·9	447	12·1	97·6	381	10·6	97·9	401	11·5	98·8	439	10·7	95·4	449	11·8	98·0

VI = (no. of liveborn/total no. born) × 100

*Total no. of liveborn.
†Average no. of liveborn/pregnant female.

Table 5. *Survival to day 4 among rats fed calcium carrageenan for three generations*

Dietary level (%)	Data on 4-day survival in generation/litter . . .																	
	F1A			F1B			F2A			F2B			F3A			F3B		
	No.†	Mean‡	SI	No.	Mean	SI	No.	Mean	SI	No.	Mean	SI	No.	Mean	SI	No.	Mean	SI
0	405	10·7	96·9	436	11·5	98·0	377	9·9	95·4	371	10·6	92·8	416	10·9	98·1	414	11·2	95·8
0·5	376	9·9	95·9	399	10·8	93·9*	401	10·3	95·9	407	10·7	96·2	417	11·0	97·9	417	11·6	97·2
1·0	423	11·1	97·7	444	11·7	95·3	345	9·3	96·1	331	9·5	97·9	405	10·4	96·4	385	10·4	96·5
2·5	425	10·6	98·2	451	11·9	96·4	429	10·7	97·1	409	10·5	89·5	420	10·8	93·5	387	10·8	93·9
5·0	395	10·4	96·3	417	11·3	93·3	351	9·8	92·1	374	10·7	93·3	425	10·4	96·8	408	10·7	90·9

SI = (no. of survivors to day 4/no. of liveborn) × 100.

†Total no. of survivors to day 4.
‡Average no. of survivors to day 4/pregnant female.
The value marked with an asterisk differs significantly from the control value: $*P < 0{\cdot}05$.

Table 6. *Survival to day 21 and weaning indexes of rats fed calcium carrageenan for three generations*

Dietary level (%)	Data on 21-day survival in generation/litter...																							
	F1A				F1B				F2A				F2B				F3A				F3B			
	No.†	Mean‡	D21I	WI	No.	Mean	D21I	WI	No.	Mean	D21I	WI	No.	Mean	D21I	WI	No.	Mean	D21I	WI	No.	Mean	D21I	WI
0	345	9·1	100·0	98·9	342	9·0	98·3	97·2	310	8·2	96·9	92·3	274	7·8	97·2	88·4	323	8·5	96·1	93·6	311	8·4	99·4	94·2
0·5	327	8·6	99·4	97·9	325	8·8	98·2	95·9	328	8·4	98·2	94·3	324	8·5	99·1	96·1	322	8·5	95·0	91·5	311	8·6	98·1	90·4
1·0	343	9·0	100·0	96·9	344	9·1	99·1	95·6	289	7·8	97·0	95·7	262	7·5	97·4	91·6	336	8·6	97·4	96·0	308	8·3	97·5	95·1
2·5	370	9·2	100·0	98·9	353	9·3	99·4	95·9	320	8·0	90·7	87·4	305	7·8	96·2	90·2	323	8·3	95·0	89·0	288	8·0	94·4	90·3
5·0	343	9·0	99·1*	98·0	327	8·8	98·5	94·0	258	7·2	89·6	86·0	286	8·2	97·3	92·0	316	7·7	96·6	90·8	294	7·7	96·4	90·7

D21I = (no. of survivors to day 21/no. alive at day 14) × 100. WI = (no. weaned at day 21/no. kept at day 4) × 100.
†Total no. of survivors to day 21.
‡Average no. of survivors to day 21/pregnant female.
The value marked with an asterisk differs significantly from the control value: $^{*}P < 0.05$.

Table 7. *Birth and weanling weights of male and female rats fed calcium carrageenan for three generations*

Dietary level (%)	Weights (g) in generation/litter...											
	F1A		F1B		F2A		F2B		F3A		F3B	
	M	F	M	F	M	F	M	F	M	F	M	F
	Birth weights											
0	6·0	5·6	6·4	6·1	6·5	6·1	6·6	6·2	6·5	6·2	6·6	6·2
0·5	6·0	5·6	6·3	5·9	6·5	6·1	6·8	6·4	6·5	6·2	6·7	6·3
1·0	6·0	5·6	6·2	5·9	6·5	6·2	6·7	6·3	6·4	6·0	6·7	6·4
2·5	6·0	5·7	6·2	5·8**	6·4	6·0	6·5	6·0	6·4	6·0	6·5	6·1
5·0	5·9	5·5	6·1	5·7**	6·4	6·1	6·5	6·2	6·2*	5·8*	6·5	6·2
	Weanling weights											
0	43·2	40·6	44·0	43·0	42·0	41·2	44·4	43·4	44·5	42·3	45·4	42·9
0·5	42·6	40·8	42·1	40·2*	42·1	39·2	46·0	43·8	41·9	40·7	44·5	41·8
1·0	42·2	40·3	39·9***	37·5***	38·8*	37·2*	45·3	43·3	40·7**	38·5***	43·5	41·5
2·5	40·6	39·0	40·7**	38·9***	37·4***	35·8***	41·8	39·1**	38·8***	37·4***	40·6***	9·2***
5·0	37·9***	36·1***	35·3***	34·5***	35·1***	34·0***	40·4*	39·4**	35·5***	34·6***	40·6***	9·5***

Values marked with asterisks differ significantly from the control value: $^{*}P < 0.05$; $^{**}P < 0.025$; $^{***}P < 0.01$.

effect on reproductive processes and only minimal effects on the viability of the foetuses. The most pronounced effect of the compound at high dietary levels was the decrease in the weight of the liveborn and the dose-related reduction in weanling weights. These decreases indicate nutritional deficiency perhaps due to the diarrhoea observed in the animals or possibly related to the liver lesions seen in some of the adults and weanlings. Since the pathological findings are not yet complete, it is difficult to ascribe a specific relationship. This will be considered in the extensive report of the pathological findings, to be published as a separate paper. There is some evidence of the degradation of very high-molecular-weight carrageenan to lower molecular weights (100,000 or less) during its passage through the intestinal tract (Pittman *et al.* 1976). If so, it is possible to speculate that the mother's milk could contain carrageenan which could lead to nutritional deficiency in the pups. Another possibility is that carrageenan may delay the start of, or interfere with, lactation so that feeding of the young is impaired.

The decreased weights in this study differ from the findings obtained in a 6-month study in rats (from weaning to maturity) on calcium carrageenan that was heat-sterilized and mixed with skim milk before being added to the diet (Tomarelli, Tucker, Bauman, Savini & Weaber, 1974). In the latter study, no adverse effects on growth rate or dietary efficiency were seen at dose levels up to 4%. It is possible that binding of the carrageenan to casein in milk minimized any adverse effects. It is also possible to speculate that the results of Tomarelli *et al.* (1974) might have shown an effect if carrageenan had been given at the same dose levels in a multigeneration study in which the animals were exposed from conception.

In view of the decreased body weights at 1·0, 2·5 and 5·0% dietary levels of carrageenan, the no-effect level in rats can be stated as 0·5%. The concentration in use for the stabilization of milk products is approximately 0·01–0·03%, with less in infant formulas (Tomarelli *et al.* 1974), while considerably greater quantities are used in meringues and puddings (Klose & Glicksman, 1972). The average per capita consumption of carrageenan has been estimated to be 23–29 mg/day (FDA, unpublished data, 1973).

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LONG-TERM EFFECTS OF CALCIUM CARRAGEENAN IN RATS.—II. EFFECTS ON FOETAL DEVELOPMENT

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Abstract—Long-term multigeneration effects of the dietary intake of calcium carrageenan were measured in a three-generation reproduction and teratology study in Osborne–Mendel rats. Dietary levels of 0·5, 1·0, 2·5 or 5·0% were ingested throughout the study. Developmental effects were studied in the F2C and F3C litters. No dose-related effect on maternal weight gain was observed. The average numbers of corpora lutea, implantations and early or late deaths, and the average percentage resorptions per litter showed no dose-related differences. No specific external, skeletal or soft-tissue anomaly could be correlated with dosage.

INTRODUCTION

Although high-molecular-weight carrageenan has been and continues to be used extensively in food, few reports are available on its possible foetotoxic properties. Frohberg, Oettel & Zeller (1969) reported a slight decrease in the percentage of live foetuses (9·1% compared with 9·5% in controls) and the occurrence of one cleft palate in 82 live offspring after ip injection of 1% aqueous carrageenan mucilage (type unspecified) into pregnant mice on days 11–15 of gestation.

In a study on the intubation of high-molecular-weight calcium κ,λ-carrageenan in corn oil suspension into pregnant mice, rats, rabbits and hamsters, it was reported that the average number of resorptions increased and foetal weight decreased in mice and rats, but no differences appeared in rabbits and hamsters (Food and Drug Research Laboratories, Inc., 1973a). In a study of high-molecular-weight sodium κ,λ-carrageenan in corn oil suspension intubated into the same species, foetal weight was decreased in mice and rats, and there was an increased number of resorptions in mice, but hamsters and rabbits showed no compound-related foetal effects (Food and Drug Research Laboratories, Inc., 1973b).

Because of the paucity of information on the effect of continuous ingestion of κ,λ-carrageenan on reproduction and foetal development, a combined three-generation reproduction and teratology study was performed. The results of the reproduction study are reported separately (Collins, Black & Prew, 1977), and the embryological effects of the compound are reported here. Animals from the second and third generations, the F2C and F3C litters, respectively, were killed for the evaluation of embryological effects.

EXPERIMENTAL

Materials and animals. Calcium κ,λ-carrageenan, Gelcarin-HMR (Lot No. 102012, Marine Colloids, Inc., Rockland, Maine) was mixed with Purina Rat Chow (Ralston-Purina Co., Inc., St. Louis, Mo.) at levels of 0·5, 1·0, 2·5 or 5·0% (5000–50,000 ppm). The major components were: alcohol precipitables, 97·57%; sulphate ester, 24·43%; free sulphate, 2·44%; 3,6-anhydro-*d*-galactose, 22·98%; water, 9·41%; potassium, 5·95%; calcium, 2·51%; sodium, 1·24%; chloride, 1·02%; nitrogen, 0·21%; magnesium, 0·04%. The viscosity of a 1·5% solution was 195·0 cP at 75°C. Osborne–Mendel rats were obtained from the FDA breeding colony. The carrageenan-containing diets were fed to the experimental animals throughout the study, and unadulterated rat chow was fed to the control animals. The parental animals were F1B and F2B, and the offspring were F2C and F3C respectively, of a three-generation reproduction study (see Fig. 1 in Collins *et al.* 1977).

Experimental procedure. Males were placed with females at approximately 5.30 pm and the presence of sperm was checked by vaginal smears the following morning. Non-sibling crosses were made with randomly chosen animals. The day on which sperm were found was designated day 0 of gestation. On day 20, the females were killed by CO_2 inhalation, and the uterus was opened and examined for the presence of resorption sites. Deciduomas, brownish implantation sites without placentas, were called early deaths, and implantation sites with placentas and with complete but non-viable foetuses that were of subnormal size, showed retarded development or were in a macerated condition were classed as late deaths, according to the terminology of Bateman & Epstein (1971) and the MARTA Committee on Terminology (1969). The live foetuses were removed, sexed, weighed and examined for gross external malformations under a dissecting microscope. Corpora lutea were counted under a dissecting microscope. Approximately one half of the foetuses from each litter at each dose level were examined for skeletal anomalies after being fixed in ethyl alcohol, cleared and stained with alizarin red (Dawson, 1926). The remaining foetuses were fixed in Bouin's solution and sectioned according to the method of Wilson (1965) in order to detect internal malformations of the soft tissue.

Table 1. *Maternal and reproductive data for rats fed calcium carrageenan*

Generation/litter	Dose level (%)	No. of pregnant females examined	Maternal body weight gain (g)†	Autopsy findings (mean/dam): Corpora lutea	Autopsy findings (mean/dam): Implantations	Autopsy findings (mean/dam): Resorptions: Early	Autopsy findings (mean/dam): Resorptions: Late	Autopsy findings (mean/dam): Viable foetuses	Resorptions per litter (%)	Preimplantation loss (%)	Females with resorptions (%): One or more	Females with resorptions (%): Two or more
F2C	0	30	119·8	14·0	11·9	1·0	0·00	10·9	9·7	14·8	56·7	21·3
	0·5	37	115·6	14·0	12·2	1·4	0·05	10·7	13·3	12·9	75·7	29·7
	1·0	34	102·6**	13·5	11·4	1·6	0·00	9·8	16·1	15·9	76·5	32·3
	2·5	36	119·7	13·9	13·0	1·4	0·03	11·7	10·7	5·8	69·4	38·9
	5·0	29	116·9	13·8	13·1	1·1	0·03	11·9	10·0	5·0**	58·6	31·0
F3C	0	34	129·3	14·3	13·1	1·1	0·00	12·0	10·1	8·8	55·9	26·5
	0·5	33	126·3	14·5	13·4	1·5	0·00	11·9	10·9	7·5	63·6	42·4
	1·0	32	115·1*	14·1	12·2	1·3	0·06	10·9	12·7	13·1	62·5	34·4
	2·5	35	122·9	14·2	12·4	1·1	0·03	11·3	9·8	12·6	65·7	31·4
	5·0	37	122·0	14·6	12·1	1·1	0·05	11·0	11·6	17·2	64·9	29·7

†Based on means adjusted to initial weight.
Values marked with asterisks differ significantly from the control value: *$P < 0·05$; **$P < 0·02$.

Statistical analysis. The data on the numbers of corpora lutea per dam, of implantations per dam and of viable foetuses per litter and on the daily food consumption of the dams were analysed for statistical significance by the two-tailed *t* test. An analysis of covariance was used to test the data on maternal body-weight gain, which was adjusted for day 0 or initial weight. The adjusted means were then analysed by a least significant difference (LSD) test. The data on the number of resorptions per litter were transformed by use of the Freeman–Tukey arc-sine transformation for binomial proportions (Mosteller & Youtz, 1961), followed by a two-tailed *t* test between the control and each dose level. The number of litters with one or more resorptions and the number of litters with one or more abnormalities were analysed by the two-tailed chi-square test. The data on foetuses with one or more abnormalities per litter were transformed utilizing the Freeman–Tukey square root transformation for Poisson distribution (Mosteller & Youtz, 1961) followed by a *t* test. Analysis of variance and an LSD test were used for data on foetal body weight and crown-rump length.

RESULTS

No dose-related effect on body-weight gain was observed in mothers of either F2C or F3C litters, although a decrease was observed at the 1% level in both generations (Table 1). The average daily calcium carrageenan consumption of mothers of the F2C animals was 309, 609, 1563 and 3184 mg/kg for the 0·5, 1·0, 2·5 and 5·0% levels, respectively. For the same dietary levels in mothers of F3C animals, daily calcium carrageenan consumption was 290, 592, 1524 and 3060 mg/kg.

The average numbers of corpora lutea, implantations and early or late deaths, and the percentages of resorptions per litter showed no dose-related differences (Table 1). The percentage of preimplantation loss was decreased at the two highest dietary levels in F2C compared with control values (and was statistically significant at the 5% level) but the difference was not biologically significant because of variations in control values (14·8 and 8·8 for F2C and F3C, respectively). The percentage of females with one or more resorptions was increased at the 0·5 and 1·0% levels in F2C but was not affected in F3C. The percentage of females with two or more resorptions was increased at all levels over the control, but no value was statistically significant. None of the litters was totally resorbed.

Both male and female foetuses of treated groups were generally heavier and longer than the control animals, but the increases were greater in males (Table 2). The weight of male foetuses was increased significantly over that of the controls at all four dietary levels in F2C and at the two highest dietary levels in F3C. The increase in foetal weight was less pronounced for females; there were significant increases in F2C at the 2·5 and 5·0% dietary levels, but none in F3C. The crown–rump lengths for males increased significantly in F2C at the 0·5, 2·5 and 5·0% levels, and at the two highest dietary levels in F3C. For females of F2C, a single statistically significant increase occurred at the 5·0% dose level. The relatively small differences in weight and length were significant because of the large number of animals (Table 1).

No external abnormality nor specific type of soft-tissue anomaly was correlated with treatment (Table 3). There was no apparent dose relationship in the average number of soft-tissue abnormalities per litter (Table 4). The average numbers of foetuses with soft-tissue anomalies per litter did not differ significantly from the control value in F2C, but was significantly decreased at the 1 and 5% dietary levels in F3C. There was no relationship with carrageenan intake when values were analysed as a percentage of total foetuses. Approximately one half of the control litters contained abnormal foetuses, and values for experimental groups were not significantly different at any dietary level.

No specific skeletal variation could be correlated with dosage (Table 5). The incidence of sternebral variations is considered separately in Table 6. Two or three sternebral variations per litter were found at each dietary level, and approximately one third to one half of the foetuses were affected. A decrease in the average number of foetuses affected per litter in F2C at the 1% dose level was statistically signifi-

Table 2. *Weight and length of foetuses of rats fed calcium carrageenan*

Generation/ litter	Dose level (%)	Mean values/litter: Foetal body weight (g)		Crown–rump length (mm)	
		Male	Female	Male	Female
F2C	0	3·8	3·6	38·5	37·3
	0·5	4·0*	3·7	39·3*	38·0
	1·0	3·9*	3·7	39·2	37·9
	2·5	4·0***	3·8*	39·3*	38·0
	5·0	4·1***	3·9***	39·8***	38·7***
F3C	0	3·7	3·5	38·3	37·5
	0·5	3·7	3·5	38·5	37·6
	1·0	3·8	3·6	38·6	37·8
	2·5	3·8*	3·6	39·1**	38·0
	5·0	3·9***	3·7	39·2***	38·2

Values are means for the numbers of litters indicated in Table 1 and those marked with asterisks differ significantly from the control value: $^{*}P < 0·05$; $^{**}P < 0·02$; $^{***}P < 0·01$.

Table 3. *Incidence of specific external and soft-tissue abnormalities in foetuses of rats fed calcium carrageenan*

	Incidence* in generation/litter...									
	F2C					F3C				
Abnormality — Dose level (%)...	0	0·5	1·0	2·5	5·0	0	0·5	1·0	2·5	5·0
External examination										
No. of foetuses examined	328	397	333	420	346	408	393	349	395	406
Haemorrhage	3 (0·9)	2 (0·5)	6 (1·8)	5 (1·2)		22 (5·4)	14 (3·6)	10 (2·9)	15 (3·8)	7 (1·7)
Club foot			1 (0·3)							
Oedema						1 (0·2)				
Soft-tissue examination										
No. of foetuses examined	174	209	170	216	176	207	195	174	200	205
Hydroureter, moderate	6 (3·4)	17 (8·1)	8 (4·7)	9 (4·2)	9 (5·1)	15 (7·2)	12 (6·1)	6 (3·4)	11 (5·5)	10 (4·9)
severe	1 (0·6)									
Hydronephrosis, moderate	6 (3·4)	20 (9·6)	9 (5·3)	7 (3·2)	8 (4·5)	21 (10·1)	13 (6·7)	7 (4·0)	11 (5·5)	7 (3·4)
severe			2 (1·2)	1 (0·5)						
Ectopic kidney	2 (1·1)	1 (0·5)	1 (0·6)	2 (0·9)	2 (1·1)	6 (2·9)	1 (0·5)	3 (1·7)	2 (1·0)	1 (0·5)
Haemorrhage	10 (5·7)	9 (4·3)	5 (2·9)	2 (0·9)	9 (5·1)	12 (5·8)	20 (10·2)	5 (2·9)	18 (9·0)	11 (5·4)
Ectopic adrenal	1 (0·6)									
Ectopic ovary						1 (0·5)				
Enlarged ventricle						1 (0·5)				1 (0·5)
Pitted kidney surface						1 (0·5)			1 (0·5)	

*No. of foetuses affected and, in parentheses, no. of malformed foetuses expressed as a percentage of no. examined.

Table 4. *Incidence of soft-tissue abnormalities in foetuses of rats fed calcium carrageenan*

		Soft-tissue abnormalities		Foetuses with one or more soft-tissue abnormalities			Litters with one or more abnormal foetuses	
Generation/litter	Dose level (%)	No.	Mean/litter	No.	Mean/litter	% of total foetuses	No.	%
F2C	0	26	0·87	22	0·73	12·64	15	50·00
	0·5	47	1·27	32	0·86	15·31	22	59·46
	1·0	25	0·73	18	0·53	10·59	11	32·35
	2·5	21	0·58	14	0·39	6·48	11	30·55
	5·0	28	0·96	20	0·69	11·36	17	58·62
F3C	0	57	1·68	45	1·32	21·74	22	54·70
	0·5	46	1·39	39	1·18	20·00	22	66·67
	1·0	21	0·66	15	0·47**	8·62	14	43·75
	2·5	43	1·23	33	0·94	16·50	19	54·28
	5·0	30	0·81	24	0·65*	11·71	19	51·35

Values marked with asterisks differ significantly from the control value: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

Abnormality	Dose level (%)...	F2C 0	F2C 0·5	F2C 1·0	F2C 2·5	F2C 5·0	F3C 0	F3C 0·5	F3C 1·0	F3C 2·5	F3C 5·0
		Incidence* in generation/litter...									
No. of foetuses examined		149	185	162	203	166	199	198	175	193	200
Sternebrae: incomplete		57 (38·2)	59 (31·9)	39 (24·1)	42 (20·7)	53 (31·9)	48 (24·1)	42 (21·2)	32 (18·3)	34 (17·6)	41 (20·5)
missing		18 (12·1)	26 (14·0)	23 (14·2)	14 (6·9)	18 (10·8)	27 (13·6)	29 (14·6)	23 (13·1)	26 (13·5)	23 (11·5)
bipartite		4 (2·7)	3 (1·6)	3 (1·8)	2 (1·0)	2 (1·2)	4 (2·0)	7 (3·5)	5 (2·8)	7 (3·6)	4 (2·0)
malaligned		12 (8·0)	7 (3·8)	8 (4·9)	15 (7·4)	9 (5·4)	6 (3·0)	5 (2·5)	3 (1·7)	8 (4·1)	8 (4·0)
Poorly ossified cranial bones		9 (6·0)	8 (4·3)	7 (4·3)	14 (6·9)	5 (3·0)	24 (12·1)	20 (10·1)	19 (10·8)	17 (8·8)	8 (4·0)
Hyoid bone: poorly ossified		3 (2·0)			1 (0·5)		3 (1·5)	2 (1·0)	1 (0·6)		4 (2·0)
missing		2 (1·3)			1 (0·5)	1 (0·6)	1 (0·5)	1 (0·5)			2 (1·0)
bipartite		1 (0·7)			3 (1·5)	2 (1·2)	1 (0·5)	1 (0·5)	1 (0·6)	1 (0·5)	
Bipartite centrum		1 (0·7)					1 (0·5)	2 (1·0)		1 (0·5)	1 (0·5)
Poorly ossified vertebrae		1 (0·7)						2 (1·0)			
13th rib bud			1 (0·5)	1 (0·6)	1 (0·5)		1 (0·5)		2 (1·1)		1 (0·5)
14th rib bud		10 (6·7)	13 (7·0)	10 (6·2)	21 (10·3)	14 (8·4)	22 (11·0)	15 (7·6)	12 (6·8)	33 (17·1)	15 (7·5)
Ribs: poorly ossified					1 (0·5)	1 (0·6)			1 (0·6)	1 (0·5)	1 (0·5)
fused		1 (0·7)									
wavy					2 (1·0)	4 (2·4)	9 (4·5)	1 (0·5)	4 (2·3)	5 (2·6)	
Poorly ossified phalanges		10 (6·7)	7 (3·8)	3 (1·8)	5 (2·5)	4 (2·4)	16 (8·0)	19 (9·6)	21 (12·0)	13 (6·7)	7 (3·5)
metatarsals					1 (0·5)			1 (0·5)			1 (0·5)
ischium								1 (0·5)			1 (0·5)
pubis								3 (1·5)			1 (0·5)
metacarpals											1 (0·5)

*No. of foetuses affected and, in parentheses, no. of malformed foetuses expressed as a percentage of no. examined.

Table 6. *Incidence of sternebral variations in foetuses of rats fed calcium carrageenan*

Generation/ litter	Dose level (%)	Sternebral variations		Foetuses with one or more sternebral variations			Litters with one or more abnormal foetuses	
		No.	Mean/litter	No.	Mean/litter	% of total foetuses	No.	%
F2C	0	91	3·03	70	2·33	46·98	28	93·33
	0·5	95	2·57	75	2·03	40·54	31	83·78
	1·0	73	2·15	59	1·73*	36·42	25	73·53
	2·5	73	2·03	63	1·75	31·03	28	77·78
	5·0	82	2·83	70	2·41	42·17	21	72·41
F3C	0	85	2·50	71	2·09	35·68	29	85·29
	0·5	83	2·51	69	2·09	34·85	28	84·85
	1·0	63	1·97	53	1·66	30·28	22	68·75
	2·5	75	2·14	63	1·80	32·64	27	77·14
	5·0	76	2·05	66	1·78	33·00	30	81·08

The value marked with an asterisk differs significantly from the control value: $^{*}P < 0·05$.

cant, but was not considered to be biologically significant.

The overall incidence of other skeletal variations is presented in Table 7. The average number of skeletal variations in control animals was 1·27 and 2·29 for F2C and F3C, respectively. Both the average number of foetuses per litter affected and the percentage of total foetuses affected were slightly higher in F3C than in F2C. The average number of foetuses with one or more abnormalities per litter was significantly decreased at the 5·0% dose level of F3C, probably because of the high control value. The affected foetuses were widely dispersed among the litters, and no clumping was apparent. The significant differences at the 0·5 and 5·0% levels in F3C were not considered biologically significant.

DISCUSSION

The decrease in maternal body-weight gain at the 1% level in F2C may be correlated with a significant decrease ($P < 0.01$) in food consumption for days 0–20 at this dietary level (21·9 g/day at 1% v. 23·4 g/day for controls). However, this cannot be the explanation for the low body-weight value in F3C since there was no significant difference in the food consumption of this group (22·3 g/day at 1% v. 22·7 g/day for controls).

In the study by Frohberg *et al.* (1969), administration of 1% carrageenan to pregnant mice on days 11–15 produced a slight decrease in the percentage of live foetuses (9·1% v. 9·5% for the control group) but no other adverse effects were observed except for a cleft palate in one of the pups. In two experiments, pregnant mice were intubated with calcium κ,λ-carrageenan in a corn oil suspension in doses of 0, 10, 45, 470 or 900 mg/kg (Food and Drug Research Laboratories, Inc., 1973a). In the first study only, the number of live foetuses at 900 mg/kg was significantly decreased and the number of dams with one or more resorptions per litter was significantly increased. Both studies showed differences in the average number of resorptions per implantation and in average foetal weight after dosage with 900 mg/kg. In a similar study with pregnant rats (Food and Drug Research Laboratories, Inc., 1973a) given calcium κ,λ-carrageenan in a corn oil suspension in doses of 0, 40, 100, 240 or 600 mg/kg, the number of live foetuses was significantly decreased at 600 mg/kg in the first experiment but not in the second, and the number of dams with two or more resorptions per dam was significantly increased at 600 mg/kg in the second study only. Average foetal weight was significantly decreased at 600 mg/kg in both experiments. Two similar studies in hamsters and rabbits at dose levels of 0–600 mg/kg and 0–260 mg/kg, respectively, showed no significant differences in any of the parameters measured (Food and Drug Research Laboratories, Inc., 1973a).

In contrast to these adverse effects after intubation of mice and rats, feeding carrageenan to rats at dietary levels of 1·0 or 5·0% on days 6–15 of pregnancy and to hamsters on days 6–10 produced no significant differences from control values (FDA, unpublished data, 1973). In terms of foetotoxicity, both generations of the dietary study presented here appear to agree more closely with results of the dietary studies on rats and hamsters than with the results of the intubation study.

In teratology studies, embryotoxicity is usually manifested by an increase in resorptions, a decrease in foetal weight and an increase in the incidence of skeletal variations and soft-tissue malformations. By these criteria, carrageenan ingested in the diet does not appear to be embryotoxic.

It is puzzling to note the increase in foetal body weight and crown–rump length in the experimental animals compared with those of the controls when the animals are taken by Caesarean section 24 hr before normal littering. These increases appear to reflect a chance occurrence; we can find no experimental cause for it. These results are in marked contrast to the pattern of decreased weights noted in the experimental animals at birth (and shown in Table 6, Collins *et al.* 1977), especially at the two highest dose levels.

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Table 7. *Incidence of skeletal variations (excluding sternebral) in foetuses of rats fed calcium carrageenan*

Generation/ litter	Dose level (%)	Skeletal variations		Foetuses with one or more skeletal variations			Litters with one or more abnormal foetuses	
		No.	Mean/litter	No.	Mean/litter	% of total foetuses	No.	%
F2C	0	38	1·27	25	0·83	16·78	15	50·00
	0·5	29	0·78	27	0·73	14·59	16	43·24
	1·0	21	0·62	21	0·62	12·96	15	44·12
	2·5	50	1·39	40	1·11	19·70	19	52·78
	5·0	31	1·07	21	0·72	12·65	11	37·93
F3C	0	78	2·29	55	1·62	27·64	29	85·29
	0·5	68	2·06	45	1·36	22·73	20	60·61*
	1·0	61	1·91	45	1·41	25·71	20	62·50
	2·5	71	2·03	56	1·60	29·01	22	62·86
	5·0	43	1·16	30	0·81**	15·00	19	51·35**

Values marked with asterisks differ significantly from the control value: $^*P < 0.05$; $^{**}P < 0.01$.

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SOME OBSERVATIONS ON THE DETERMINATION OF MONOMER RESIDUES IN FOODS*

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Abstract—The effects of various experimental parameters on the determination by headspace gas chromatography of residues of vinyl chloride and of acrylonitrile in foods were examined. Preliminary investigations on the choice of solvent, the effects of varying duration and temperature of storage and the ratio of headspace to solvent volume are reported. Headspace gas chromatography was used to follow the migration of vinyl chloride from PVC into orange drink, wine, olive oil and water.

INTRODUCTION

Polyvinyl chloride (PVC) is widely used as a packaging material for food. In the USA, some 300 million pounds of PVC resin were manufactured in 1973 for the production of food-packaging products alone (Schaffner & Lombardo, 1975); only polyethylene was more extensively used for this purpose. Both vinyl chloride (VC) homopolymers and copolymers of VC with other monomers are used. During the manufacture of rigid (unplasticized) PVC products in particular, some residual VC monomer is trapped in the bulk material and remains unpolymerized. In certain conditions this residue may migrate from the container into the food. Such migration was first observed when significant organoleptic differences between spirits packed in PVC and those packed in glass were traced to the presence of VC at the mg/kg level after storage (Schaffner & Lombardo, 1975).

VC was not thought to be dangerous to human health until 1974, when its association with a rare form of liver cancer amongst workers at PVC plants was first disclosed (*Chemical Engineering News*, 1974). Animal test data (Maltoni & Lefemine, 1975) derived from the inhalation of high levels of VC gas lent support to these findings, and further studies at lower levels using alternative modes of administration are now underway. Pending the completion of this work, action by regulatory authorities has been initiated to ensure safe conditions at work and to minimize any hazard from environmental exposure.

Effective legislation to control the amounts of VC monomer in containers, in food and in the atmosphere necessitates sensitive and reliable methods for the detection and determination of very low levels of VC. Such methods must be applicable to plastics at the mg/kg level and to foods at the μg/kg level. Headspace gas chromatography (HSGC) has been used in this work by several groups (Steichen, 1976; Williams, 1976) whilst methods using the Hall detector (Ernst & van Lierop, 1975) and mass fragmentography (Rosen, Morano, Pareles, Giacin & Gilbert, 1975) have also been described.

Following the emergence of the VC problem, interest and concern has turned to other monomers, similar in chemical structure to VC and also used in the manufacture of materials and articles for food-contact use. Polyacrylonitrile is used industrially for filter cloths and so might come into contact with food. Additionally, acrylonitrile (AN) is used in conjunction with butadiene and styrene in a wide range of materials containing different proportions of the three monomers. Applications in food packaging include injection-moulded trays for carrying bread and cakes, and tubs for margarine (Briston & Katan, 1974). Although AN has a higher boiling point (75°C) than VC (−13·5°C), the HSGC technique has been used successfully for it by Steichen (1976).

Because of the interest in methods likely to be associated with EEC directives, a study of analytical methods based on HSGC for the determination of VC, AN and other monomers, both in plastics materials themselves and in foods, has commenced. Initially, the objective was to investigate a number of parameters that might affect the analytical determination. This paper presents the results of initial work on the choice of solvent, distribution of monomer between the headspace and the liquid, and effects of duration and temperature of storage. Preliminary investigations were carried out using solutions of VC and AN. Some early results on the migration of VC into foods are also reported.

EXPERIMENTAL

All results reported were obtained using a Perkin Elmer F40 gas chromatograph fitted with a FID detector using 0·125 in. stainless-steel columns packed with 20% Carbowax 1540 or 20M on 60/85 mesh Chromosorb W; column lengths varied from 2·5 to 3·5 m. Gas flows were adjusted experimentally to produce optimum detector response.

Pure VC was obtained from Matheson Gas Products via British Oxygen Co. Ltd., London, and pure AN (Koch-Light, Puriss Grade) was obtained from Koch-Light Laboratories Ltd., Colnbrook,

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Bucks. VC standard solutions were prepared by dilution of chilled saturated solutions and were kept in a refrigerator. Stock solutions were standardized by titration against standard thiosulphate solution after bromination and then used to prepare working standard solutions by further dilution. Solutions of AN were obtained by dilution and were stored at laboratory temperatures. The solvents used were methyl ethyl ketone (MEK; Koch-Light Laboratories Ltd.; Puriss grade), dimethylformamide (DMF; Fisons Chemicals Ltd., Loughborough, Leics.; SLR grade), dimethylacetamide (DMA; Koch-Light Laboratories Ltd.; Puriss grade) and Pure British Spirit (PBS; 95% ethanol, v/v). Experiments were carried out in F40 vials (approximately 23 ml in volume), each sealed with a septum of silicone rubber.

To determine the effect of various factors on VC distribution, VC (b.p. −13·5°C) solutions in MEK (5 ml containing 1·05 μg VC) or water (5 ml containing 0·21 μg VC) were stored in the F40 vials at 30 or 40°C. The headspace gases were analysed at regular intervals until full equilibrium had been attained and had remained stable for at least 5 hr. For AN (b.p. 75°C), the temperatures used were 60 and 80°C and the solvents were DMF, DMA and water (5 ml of each containing 19·6, 9·0 and 1·0 μg AN, respectively).

The effect of altering the ratio between the volume of the headspace and the volume of VC solution was also studied using four different solvents: water, MEK, PBS and DMA. A constant weight of VC (1·05 μg) was added to varying volumes of liquid (5–20 ml) leaving a headspace of 18·5–3·5 ml in the F40 vials.

Samples of orange drink, red wine, olive oil and water, which had not previously been in contact with PVC, were poured into unused PVC bottles of capacity 1 litre and approximate mass 46 g, leaving a minimum air-space above the liquid. The bottles were then tightly sealed with screw caps and stored at room temperature in the dark. At approximately monthly intervals the bottles were opened and 5 ml portions of the liquid were removed for analysis by HSGC. The 5 ml samples of liquid were sealed in F40 vials and equilibrated at 40°C for 2 hr and the headspace gas was injected onto the GC column using the automatic facility of the F40 instrument. After analysis the liquid was returned to the appropriate bottle and the cap was replaced. After a 10-month storage period the bottles were emptied and portions of plastic cut from the base of each bottle were examined for residual VC content by dissolving 0·25 g in MEK followed by analysis using HSGC.

In a parallel series of experiments the same fluids, to which varying weights of VC had been added, were gently poured into open glass jars for storage at room temperature. A 250-ml volume of liquid containing from 0·04 ppm VC in the orange drink to 1·9 ppm VC in the vegetable oil was used and the surface area exposed to the atmosphere was 80 cm^2. Aliquots (5 ml) were taken from the liquids at intervals and sealed in F40 vials prior to HSGC analysis, to determine the loss of VC to the atmosphere over 24 hr.

Table 1. *Changes of VC content of headspace gases with storage time using water or MEK as solvent*

Duration of storage (hr)	Solvent...	VC peak area using HSGC arbitrary units: Water	MEK
0·5		7069	3841
1		9659	3829
2		10075	4012
3		10072	3992
4		9379	4016
5		9374	4080
6		9237	4220
7		9451	4016

RESULTS AND DISCUSSION

In any method based on HSGC the distribution of the analyte between the headspace and the solid or liquid phases in the container is of paramount importance and is likely to be dependent on a number of related factors. Whilst the effect of temperature is readily predicted, other factors are more difficult to assess. In our preliminary work, we were interested in the rate of attainment of equilibrium in the headspace using different solvents and different temperatures.

For VC, equilibrium is attained more rapidly in MEK than in water. In the latter solvent at 30°C, full equilibrium is attained only after about 2 hr (see Table 1); at 40°C, the equilibrium time can be as short as 30 min. At 30°C, VC is of the order of ten times more soluble in MEK than in water, judged from peak height measurements of headspace gases. After attaining equilibrium, the VC content of the headspace is stable for at least 5 hr.

For AN at 60 or 80°C, equilibrium is achieved, either in water or DMF, in less than 30 min and very little change is observed over a period of 7 hr (Fig. 1). However, when the same experiments were repeated using DMA as solvent, at 80°C the concentration of AN in the headspace fell rapidly for 2 hr, beginning about 1 hr after reaching equilibrium (Fig. 2). This effect has been observed many times in our laboratories but the reason is not yet clear. It has only been observed in DMA, and is not concentration-dependent within the range 7–73 μgAN. The rate

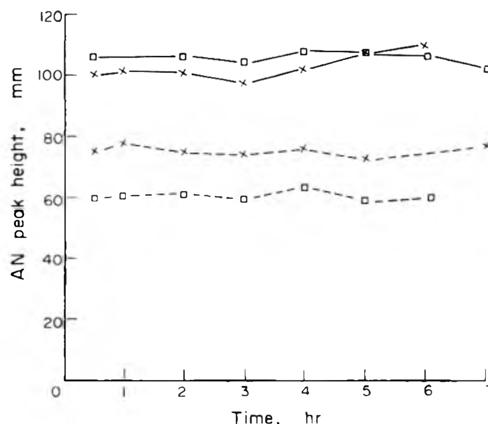

Fig. 1. The effect of temperature on changes in the AN concentration of headspace gases with storage time, using water at 60°C (---□---) and 80°C (—□—), and DMF at 60°C (---×---) and 80°C (—×—).

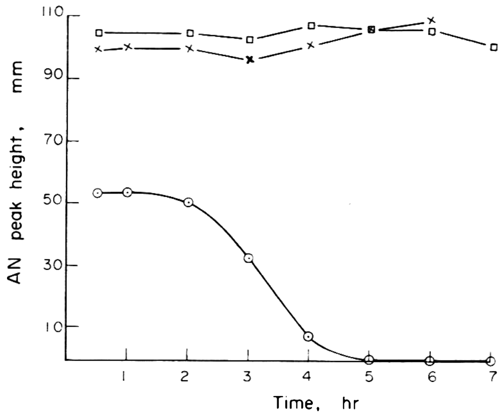

Fig. 2. The effect of solvent on changes in the AN concentration of headspace gases with storage time at 80°C using water (—□—), DMF (— x —) or DMA (—⊙—).

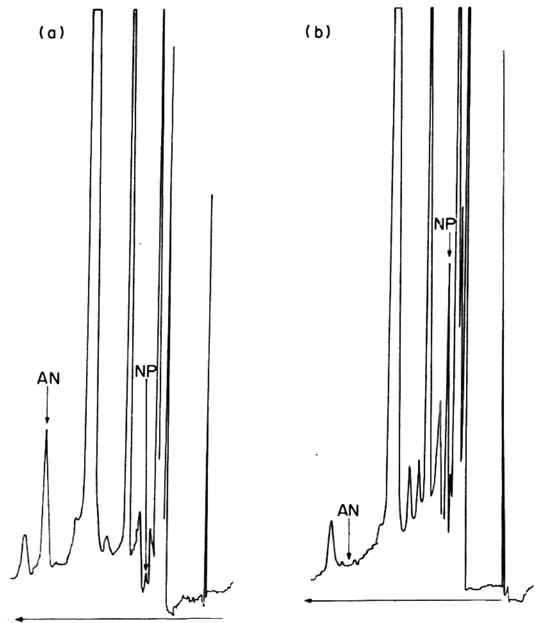

Fig. 3. Headspace gas chromatogram showing AN peaks using DMA as solvent at 80°C (a) after 30 min equilibration and (b) after 7 hr.

of disappearance varies with temperature: up to its boiling point AN is stable in DMA (at 60°C AN peak heights were constant over a 7 hr period), but at temperatures of 80 and 90°C the concentration rapidly falls to zero, the fall being more rapid at 90 than at 80°C. The disappearance of the AN peak was accompanied by the simultaneous appearance of a new peak (NP) on the chromatogram (Fig. 3) at a retention time approximately one third of that of AN. One possibility is that at high temperatures, polymerization reactions occur, although the chromatographic evidence showing a peak of shorter retention time does not support this explanation. Alternatively, some reaction with impurities present in the solvent may be taking place.

In addition to the effects of solvent and temperature, the ratio of the volumes of the liquid phase and the headspace within the container may be important. The concentration of VC in the headspace at different headspace volumes is shown in Fig. 4. There is a clear distinction between aqueous and organic solvent systems which may be important in the design of a standard method for the analysis of VC in foods.

In all cases, the migration of monomer from the container into the food followed the expected pattern (Figs. 5–7). There was a steady increase in VC content of the food during the first 3 months, followed by a levelling off and, in some cases, a slow decay. Figures 5, 6 & 7 illustrate changes in the VC content

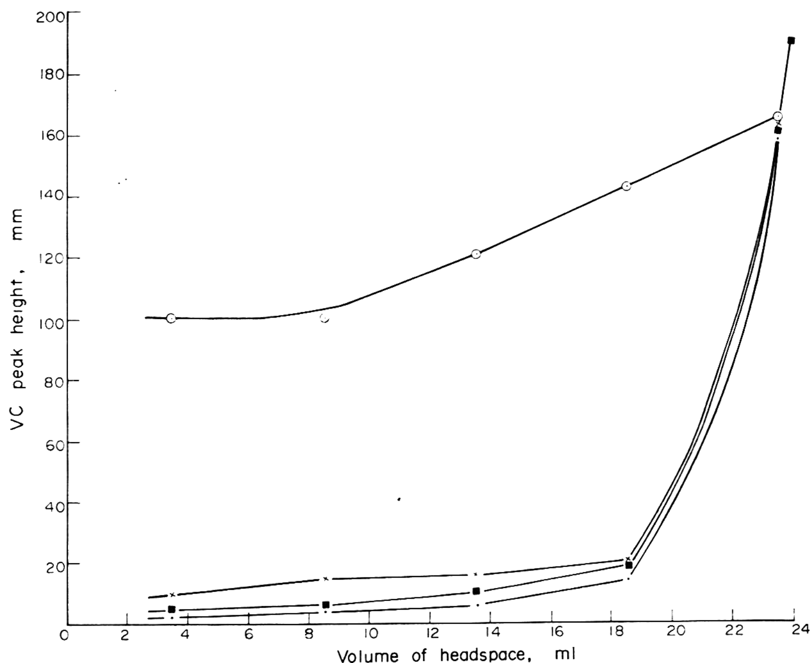

Fig. 4. Changes in the VC content of headspace gases at different ratios of headspace volume to VC solution volume using water (—⊙—), MEK (— x —), PBS (—■—) and DMA (—·—).

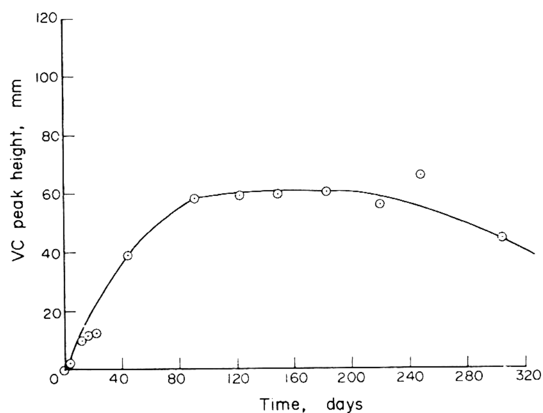

Fig. 5. The time-course of VC migration from a PVC bottle into orange drink using headspace gas chromatography to estimate VC.

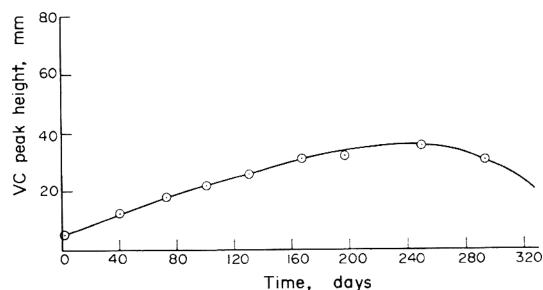

Fig. 6. The time-course of VC migration from a PVC bottle into olive oil using headspace gas chromatography to estimate VC.

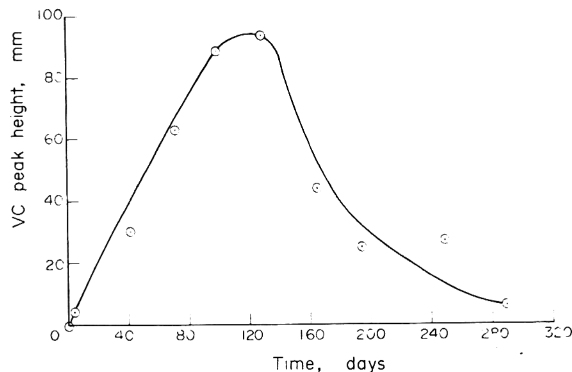

Fig. 7. The time-course of VC migration from a PVC bottle into red wine using headspace gas chromatography to estimate VC.

of the food during storage rather than absolute values. However, in Table 2 the VC content of each food at the conclusion of the study is shown, along with the corresponding residual level of VC found in the appropriate bottle. No attempt has been made to correlate the two levels since the VC content of bottles may vary to some extent from one part of the bottle to another and the residual level initially present is possibly of greater significance than the final one at the end of the study. PVC bottles currently in production contain even lower residual levels of VC monomer than those used in this study and, hence, migration of VC into the food will be correspondingly lower.

In the experiments in which the loss of VC to the atmosphere by the foods used above was determined using open glass jars, the VC content fell to half its original value over a period of 2–4 hr.

The EEC Commission has recently made a proposal to the Council for a Directive designed to limit VC in food contact materials and in food (*Official Journal of the European Communities*, 1977). The proposal includes a method based on HSGC by which VC can be determined in PVC and in food, but full experimental details of the technique are not included. Our paper highlights some of the problems that may be encountered in the determination of VC and other similar monomers using HSGC. Good analytical methods are essential both for legislative control and for a more fundamental understanding of the migration process.

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Table 2. *Vinyl chloride contents of foods and bottles at the end of a 10-month migration study*

Type of sample		VC found in food ($\mu g\ kg^{-1}$)	VC found in corresponding bottle ($mg\ kg^{-1}$)
Water	I	5	1·5
	II	11	2·7
Orange drink	I	8	2·2
	II	14	4·0
	III	7	0·8
	IV	23	3·0
Olive oil	I	14	6·9
	II	16	5·9
Red wine	I	5	6·6
	II	5	6·5

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CITRININ MYCOTOXICOSIS IN THE GUINEA-PIG

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Abstract—Citrinin was dissolved in dimethylsulphoxide–ethanol solution and administered daily for 14 days by gastric intubation to young male and female guinea-pigs. Under these conditions, the LD_{50} of citrinin was 43 mg/kg/day for both sexes. Loss of body weight was the most consistent clinical feature of citrinin toxicity. Test guinea-pigs were frequently found dead without premonitory signs. Haematological values of citrinin-dosed guinea-pigs reflected dehydration and anaemia; other clinico-pathological findings reflected renal damage. Gross lesions following treatment included swollen and discoloured kidneys, pale, mottled and friable livers, scanty intestinal contents, subserosal haemorrhage in the caecum with faecal contents of fluid consistency, and pale streaking of certain skeletal muscles. Histopathological alterations attributable to citrinin administration were most prominent in the kidneys and included degeneration and necrosis of the epithelial cells of the proximal tubules, mainly but not entirely in the convoluted portion. Other lesions apparently related to citrinin administration were bile-duct hyperplasia and hepatocyte calcification. Inflammation of the gastric mucosa was seen primarily in treated females, but mucosal ulceration of the caecum occurred in both sexes. Necrosis, with mineralization of individual fibres, was found in skeletal muscles and necrotic mononuclear cells were present in Huxley's layer of the hair-root bulbs.

INTRODUCTION

Hetherington & Raistrick (1931) isolated a yellow crystalline compound from cultures of *Penicillium citrinum* and named it citrinin. Since that initial isolation, it has been established that several species of Penicillium and Aspergillus produce citrinin (Hald & Krogh, 1973; Pollock, 1947; Scott, van Walbeek, Harwig & Fenwell, 1970; Scott, van Walbeek, Kennedy & Anyeti, 1972; Timonin & Rouatt, 1944a).

The toxicity of citrinin in mice was studied by Timonin & Rouatt (1944a) and the susceptibility of other species of laboratory animals, including guinea-pigs, rats and rabbits, was investigated by Ambrose & DeEds (1945 & 1946). Guinea-pigs that died from citrinin toxicity had marked changes in the kidneys, principally swelling, vacuolation, necrosis and desquamation of the tubular epithelium. Tubular lumina contained protein casts and a few clumps of desquamated epithelial cells (Ambrose & DeEds, 1945 & 1946). In dogs, citrinin caused an immediate but transient drop in blood pressure and tachyphylaxis after iv administration. Carlton, Sansing & Szczech (1974) characterized the clinico-pathological and histological changes produced in beagle dogs by daily ip doses of 20–40 g citrinin/kg. Renal lesions consisted of degeneration and necrosis of the tubular epithelium, most prominently in the thick segment of Henle and in the distal convoluted tubules.

In studies of a disease of swine in Denmark called mycotoxic porcine nephropathy, it was established that a citrinin- and ochratoxin A-producing strain of *P. viridicatum* induced renal lesions similar to those of the field disease (Krogh, Hald & Pedersen, 1973; Krogh, Hasselager & Friis, 1970). Swine given 20–40 mg citrinin/kg orally for as long as 42 days showed gross and histological changes only in the kidneys, which were enlarged up to five times the normal size, were yellow-grey and bore a large number of small subcapsular cysts (Friis, Hasselager & Krogh, 1969). Microscopically, the renal damage was characterized by degeneration of the proximal tubules and development of atypical basophilic epithelium containing enlarged nuclei with dark-staining chromatin.

This report describes the clinical, clinico-pathological and pathological features of citrinin mycotoxicosis in young guinea-pigs.

EXPERIMENTAL

Animals. Altogether 68 male and 68 female guinea-pigs were treated with citrinin: they were divided among the various test groups as indicated in Table 1. In addition, 11 males and nine females were used as solvent-dosed controls. The guinea-pigs (derived from the Hartley strain) were from the colony of the Purdue University School of Veterinary Medicine and were 3–8 wk old (body weights 251–529 g) when placed on test. The animals were housed in a controlled environment and provided with fresh drinking-water and commercial guinea-pig diet *ad lib*. Lettuce from a local supermarket distributor was given once weekly. Guinea-pigs were weighed and dosed daily and were observed several times daily for signs of toxicosis.

Table 1. *Experimental groups given citrinin by gastric intubation*

Group	Dose (mg/kg/day)	No. of guinea-pigs/group	
		Males	Females
I	25	10	10
II	30	5	5
III	36	15	15
IV	40	10	10
V	45	10	10
V-A	45*	5	5
VI	50	13	13

*Solution heated to 83°C during mixing.

Administration and preparation of solutions. Citrinin, prepared according to procedures previously described (Carlton *et al.* 1974), was dissolved in dimethylsulphoxide (DMSO)–50% ethanol (1:1, v/v) in appropriate concentrations for administration of the desired dose of mycotoxin in 5 ml solution/kg body weight. Doses were adjusted daily and solutions were freshly prepared every third day during the dosing period. For one trial (group V-A), the solution was heated to 83°C during mixing and was administered in a dose of 45 mg/kg. The other guinea-pigs dosed at 45 mg/kg (group V) and those given other doses (25, 30, 36, 40 and 50 mg/kg received citrinin solutions that had been mixed at room temperature. Doses were administered daily by gastric intubation for 14 days.

LD_{50} determination. This was based on the number of deaths occurring within the 14-day dosage period. The LD_{50} value was calculated using the method of moving average interpolation (Weil, 1952) with the following constants: $n = 10$, $K + 1 = 4$ and $d = 0{\cdot}0453$, where n = the number of animals dosed per treatment, $K + 1$ = the number of dose levels used (the dose levels being 36, 40, 45 and 50 mg/kg in this case) and $d = \log R$, R being the geometric factor used for the difference between successive dose levels and equalling 1·11 in this study.

Clinical pathology. At autopsy, blood was collected by cardiac puncture from animals of groups I, III and VI for determinations of packed cell volume by a standard microhaematocrit method, of total concentrations of plasma protein by the refractometer method, and of haemoglobin concentrations by a photo-electric reading system (Coulter Hemoglobinometer, Coulter Electronics, Inc., Hialeah, Fla), and for counts of total erythrocytes and leucocytes by an electronic counting device (Coulter Counter, Model Fn, Coulter Electronics, Inc.). Serum concentrations of sodium and potassium ions were determined by flame photometry and of chloride ion by the method of Cotlove, Trantham & Bowman (1958).

On termination of dosing, solvent-dosed control guinea-pigs and animals from the experimental groups I, III and VI were housed overnight in individual mesh-bottom, stainless-steel metabolism cages and urine was collected for determinations of pH, specific gravity, protein, glucose, blood, bilirubin, urobilinogen and ketones (Multistix, Ames Co., Elkart, Ind.). Urine sediments were stained with Wright's stain for microscopic examination. Portions of urine samples were dialysed for 2 hr in running tap-water in preparation for enzyme-activity determinations.

The activities of lactic dehydrogenase, isocitric dehydrogenase, glutamic–oxalacetic transaminase and glutamic–pyruvic transaminase were determined in serum and urine using commercial enzyme test systems (Boehringer Mannheim Corp., New York).

Pathology. Guinea-pigs found dead, those killed when moribund and the survivors killed at the end of the 14-day trial period were autopsied. Representative portions of most organs were fixed in 10% neutral buffered formalin, processed for paraffin sectioning and stained with haematoxylin and eosin for histopathological examination. Portions of liver and kidneys from selected test animals and controls were fixed in formol–alcohol fixative and processed by Best's carmine method for glycogen. Selected sections of kidneys were also stained by the Masson's trichrome method and by the periodic acid–Schiff (PAS) method.

RESULTS

Clinical observations

Clinical signs were seldom observed in guinea-pigs given the low doses of citrinin. However, loss of body weight, which was apparent in all the groups given citrinin, was usually seen within 24 hr of the first dose (Figs 1 & 2), although in the lower dosage groups this initial loss was subsequently recovered. Frequently guinea-pigs given 45 or 50 mg citrinin/kg/day (groups V and VI) were listless and had piloerection and an unkempt pelage by day 2 of dosing. The test animals were often found dead without deterioration of condition and their general appearance could not be correlated with impending death.

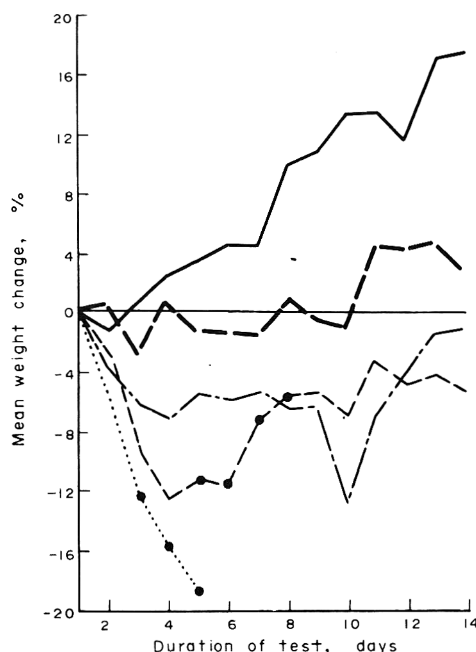

Fig. 1. Mean body-weight changes in male guinea-pigs given 0 (—), 36 (——), 40 (–·–), 45 (– –) or 50 (·····) mg citrinin/kg/day by gastric intubation for 14 days. Times at which deaths occurred are indicated (●).

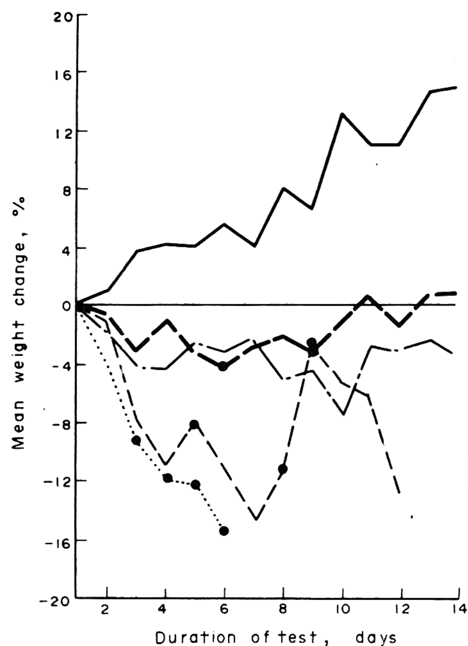

Fig. 2. Mean body-weight changes in female guinea-pigs given 0 (—), 36 (——), 40 (– – –), 45 (——) or 50 (· · · · ·) mg citrinin/kg/day by gastric intubation for 14 days. Times at which deaths occurred are indicated.

LD_{50} determination

The average survival times of male guinea-pigs in groups I–VI were 324, 336, 336, 336, 325 and 68 hr respectively. The average times for the females given the same doses were 295, 336, 313, 336, 211 and 98 hr. Most deaths (21/22 males and 22/27 females) occurred within the first 7 days of dosing. The 14-day LD_{50} of citrinin was 43·9 mg/kg/day (95% confidence interval 42·7–45·2) for male guinea-pigs and 42·9 mg/kg/day (95% confidence interval 32·5–56·8) for females. The difference in the LD_{50} values between males and females was not statistically significant.

Clinico-pathological observations

Haematology. The packed cell volume of the control guinea-pigs varied from 33 to 50% (mean 38%) while that in citrinin-dosed guinea-pigs ranged from 27 to 42% (mean 31%). The lower value in the test animals was accompanied by lower erythrocyte counts (Table 2). The leucocyte counts varied greatly among both controls (3·4–83·9 × 10^3/mm^3 blood) and test animals (2·6–51·1 × 10^3/mm^3). In the groups of citrinin-dosed guinea-pigs, mean values for total plasma proteins were moderately elevated above those of the controls, while haemoglobin levels and erythrocyte counts were slightly below the control values. Serum concentrations of K^+ were significantly higher than those of control animals, Na^+ levels were slightly increased and Cl^- levels were at the lower limits of normal. No consistent change related to treatment was found in the haematological values.

Urine analysis. Urinary pH of guinea-pigs in groups I, III and VI (Table 3) was slightly lower than that of the solvent-dosed controls. The specific gravity of the urine of citrinin-dosed guinea-pigs did not differ significantly from that of controls, although the protein levels were moderately raised. Urinary sediments and values for glucose, blood, bilirubin, urobilinogen and ketones were normal in the treated groups.

Enzyme activities. The activities of lactic dehydrogenase, isocitric dehydrogenase and glutamic–oxalacetic and glutamic–pyruvic transaminases in both serum and urine were similar to those of the solvent-dosed controls and were considered to be within the normal range (Table 3).

Autopsy observations

The organs most affected by citrinin treatment were the kidneys, caecum, liver and uterus. The kidneys of dosed guinea-pigs were either diffusely pale or diffusely dark red-brown; they were frequently swollen and turgid and the cut surfaces bulged. In both the pale and dark kidneys the cortico-medullary junction was indistinct. Pale radial streaking of the cortex was seen occasionally in the kidneys of guinea-pigs given the highest doses of citrinin.

Frequently observed in the caeca were subserosal haemorrhage, varying from petechiae to ecchymoses, and discoloured (often black to red) and fluid contents (normal caecal contents are pasty and dark green). These effects were most prominent in the guinea-pigs given the highest doses of citrinin (groups V and VI).

Table 2. *Haematological and serum-electrolyte data for guinea-pigs given citrinin in daily doses of 25–50 mg/kg*

		Mean values ± 1 SD for group			
		0 (control)	I	III	VI
Parameter	Citrinin dose (mg/kg/day)...	0	25	36	50
	No. of animals sampled...	15	9	6	4
PCV (%)		38 ± 4	30 ± 12	32 ± 7	32 ± 3
TPP (mg/100 ml serum)		4·9 ± 0·3	6·1 ± 3·4	5·6 ± 2·1	6·0 ± 1·2
Hb (mg/100 ml serum)		14·3 ± 1·6	14·1 ± 4·5	12·1 ± 1·7	10·9 ± 1·5
RBC (10^6/mm^3)		5·4 ± 0·7	5·0 ± 2·0	4·8 ± 0·9	4·7 ± 0·7
WBC (10^3/mm^3)		14·6 ± 22·2	12·0 ± 17·0	12·3 ± 16	9·7 ± 8·2
Na^+ (mequiv/litre)		116 ± 30	120 ± 8	125 ± 10·2	132 ± 7·6
K^+ (mequiv/litre)		8 ± 4	10 ± 3	10 ± 3	12 ± 3
Cl^+ (mequiv/litre)		103 ± 8	102 ± 10	97 ± 8	96 ± 9

PCV = Packed cell volume TPP = Total plasma protein Hb = Haemoglobin
RBC = Erythrocytes WBC = Total leucocytes

Table 3. *Urine analysis and enzyme activities of guinea-pigs given citrinin in daily doses of 25–50 mg/kg*

Parameter		Mean values ± 1 SD for group			
		0 (control)	I	III	VI
	Citrinin dose (mg/kg/day)...	0	25	36	50
	No. of animals sampled...	15	9	6	4
Urine*					
pH		8·3 ± 0·8	7·8 ± 1·0	7·8 ± 0·9	7·9 ± 1·1
Specific gravity		1·020 ± 0·010	1·020 ± 0·010	1·018 ± 0·010	1·018 ± 0·008
Protein		0·5 ± 0·8	0·9 ± 0·9	0·8 ± 0·6	0·6 ± 0·8
Enzymes (IU/litre)					
LDH		57 ± 108	1 ± 4	11 ± 14	26 ± 45
GOT		5 ± 4	3 ± 3	5·4 ± 5·0	6 ± 4
GPT		4 ± 4	10 ± 14	7 ± 9	5 ± 6
ICDH		13 ± 12	0	4 ± 8	0
Serum					
Enzymes (IU/litre)					
LDH		296 ± 199	231 ± 49	247 ± 63	276 ± 87
GOT		53 ± 30	49 ± 11	46 ± 9	57 ± 18
GPT		24 ± 32	11·4 ± 3·5	19 ± 22	26 ± 16
ICDH		52 ± 16	55 ± 14	57 ± 17	49 ± 12

LDH = Lactic dehydrogenase GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase ICDH = Isocitric dehydrogenase

*Urinary sediment examinations and tests for glucose, blood, bilirubin, urobilinogen and ketones showed no changes.

Lesions occurred less frequently in other segments of the gastro-intestinal tract. The contents of the stomach and intestine were sometimes scanty, indicating either inappetance or increased motility, and they were occasionally haemorrhagic in females given the highest doses of citrinin. The lymph nodes of the intestinal tract were moderately enlarged and hyperaemic in a few treated guinea-pigs.

The hepatic alterations were general paleness, pale mottling and multiple pale discrete foci. Turgid and friable livers were occasionally seen. The frequency and severity of the hepatic alterations did not increase with increasing doses of citrinin.

The more common uterine alterations were turgid thickening of the wall with or without concomitant hyperaemia and luminal haemorrhage. Three females given 25 mg citrinin/kg were pregnant and no gross abnormalities were found in the foetuses.

Pale streaking of the psoas, semimembranosus, semitendinosus and rectus femoris muscles was found in two animals of group V. Pulmonary consolidation was seen occasionally in varying degree in animals of all treatment groups and in the controls.

Histopathology

The principal histopathological findings were in the kidneys, caecum and liver.

Kidney. Degeneration and necrosis involved most frequently the epithelial cells of the proximal tubules (Fig. 3) and were characterized by nuclear pyknosis, karyorrhexis and cytoplasmic vacuolation and mineralization. These alterations were minimal to mild in animals given the lower doses of citrinin (groups I–IV). Only single or small groups of epithelial cells were necrotic in the convoluted or straight segments of the proximal tubules. Degenerative changes were seldom seen in other segments of the nephron in these low-dose groups. Increased mitotic activity in tubular epithelial cells was observed occasionally in the outer stripe of the outer zone of the medulla. The altered tubular epithelial cells had a patchy distribution; some cells were large, dark-staining and frequently multinucleate and protruded into the tubular lumen. Guinea-pigs given citrinin at the highest doses (groups V and VI) had marked to severe tubular damage. Most of the epithelial cells of the proximal tubules were necrotic and other segments, particularly those of the distal convoluted portions, contained necrotic cells. Mineralization of individual tubular epithelial cells, especially in the proximal convoluted segments, was frequent. Although the epithelial cells in these segments were severely damaged, cellular debris and desquamated epithelial cells were found in the tubular lumina only infrequently. Hyaline casts were moderately frequent in tubules of the medullary rays. Dilated cortical tubules were observed frequently in the kidneys of guinea-pigs at all dose levels but the severity was not dose related.

Liver. Lesions found in the livers of citrinin-treated guinea-pigs included focal necrosis, focal calcification and mild biliary hyperplasia. Focal hepatic necrosis was observed also in control animals and neither the severity nor the incidence of this lesion increased in the higher dosage groups. The foci of necrosis were discrete and of variable size and were most often located subcapsularly. Necrotic foci were found in all lobes and were not associated with the vessels of the portal region. Necrotic hepatocytes had pale, often vacuolated cytoplasm and pyknotic nuclei, and some were mineralized. Calcification of the periphery of necrotic foci occurred most frequently in guinea-pigs given the highest doses of citrinin; it was not found in control animals. The mineralization was intracellular and, where it was prominent, nearly all the hepato-

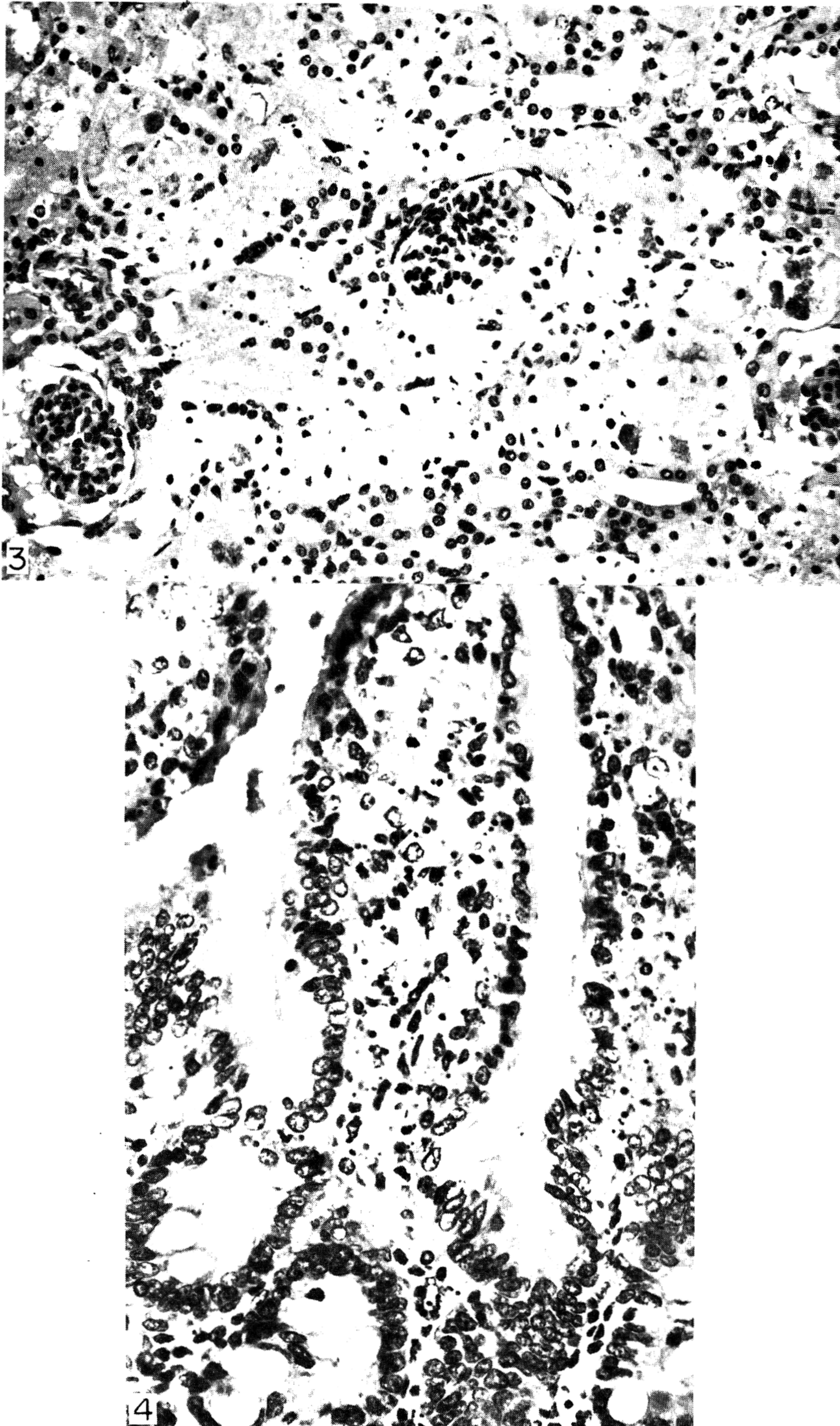

Fig. 3. Degeneration, necrosis and focal mineralization of proximal tubular epithelial cells of the kidney of a guinea-pig given 50 mg citrinin/kg/day. Haematoxylin and eosin ×224.

Fig. 4. Necrosis of leucocytes in the lamina propria and moderate oedema of the villi of the ileum in a guinea-pig given 50 mg citrinin/kg/day. Haematoxylin and eosin ×350.

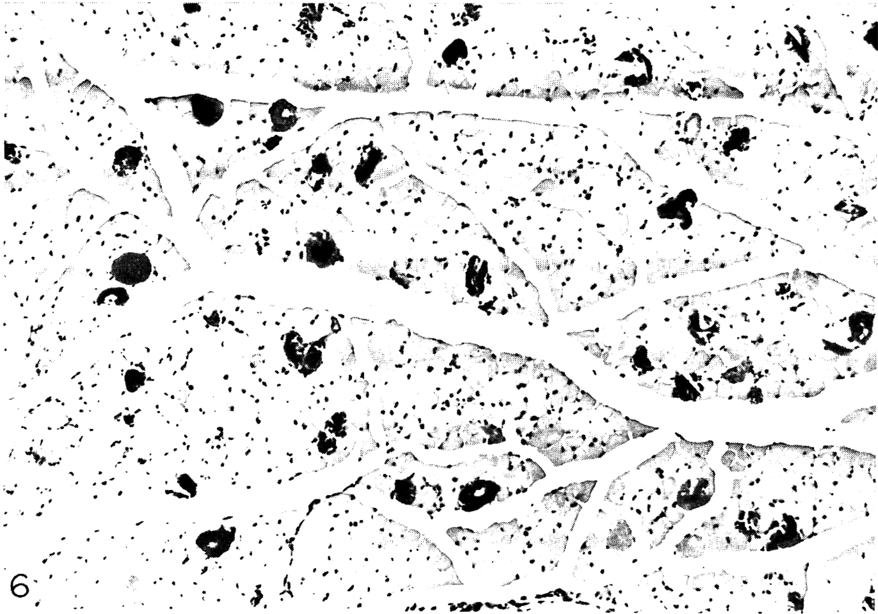

Fig. 5. Focal mucosal ulceration of the caecum of a guinea-pig given 50 mg citrinin/kg/day. Haematoxylin and eosin × 88.

Fig. 6. Focal mineralization of psoas muscle of a guinea-pig given 50 mg citrinin/kg/day. Haematoxylin and eosin × 88.

cytes surrounding a necrotic focus were calcified. Occasionally, calcified hepatocytes were dispersed throughout the parenchyma.

Hyperplasia of bile ducts was observed frequently in guinea-pigs of groups IV, V and VI. This change was usually minimal to moderate and was disseminated in several lobes. Biliary hyperplasia was most frequently seen in animals of group V and was not seen in controls.

Minimal to moderate fatty vacuolation involved the centrilobular hepatocytes of some guinea-pigs and was generalized in occasional animals in all dosage groups. Neither the severity nor the incidence was dose related. Generalized fatty change was seen in a few solvent-dosed controls. The liver from occasional guinea-pigs in all groups had an increased glycogen content. The change was not related to citrinin administration as it occurred in control as well as treated animals.

Intestine. Mucosal inflammation, necrosis and ulceration were present in various sections of the gastro-intestinal tract of the treated animals, lesions being most frequent in the caecum. Mucosal hyperaemia was common and often prominent, but localized typhlitis with infiltration of the mucosa with polymorphonuclear leucocytes was less common. Necrosis of the mononuclear leucocytes of the lamina propria of the mucosa of the duodenum, jejunum, ileum and colon was seen occasionally in most test groups (Fig. 4). Necrotic cells were often found below the normal mucosal epithelium. Focal ulceration and haemorrhage (Fig. 5) were less frequent. Necrosis of the caecal mucosa was the most consistent finding and was severe in the animals of groups V and VI. These changes were not seen in control animals.

Stomach. Lesions found in the stomach were localized hyperaemia, inflammatory-cell infiltration and dilatation of the gastric pits. Ulceration of the gastric mucosa was found only in females and was dose related.

Skin. Nuclear pyknosis and cytoplasmic vacuolation of mononuclear cells in Huxley's layer of the hair-root bulbs was frequent in animals of groups V and VI. Mitotic activity in the cells of this layer was greater in groups I–IV than in controls, but the relationship of this finding to citrinin administration was not established. No significant changes were found in other adnexal structures, the epidermis, dermis or subcutis.

Uterus. Vascular congestion of the mucosal and myometrial tunics was the most common finding in treated guinea-pigs, but the incidence and severity did not differ greatly from the occurrence in controls. Slight to moderate endometritis accompanied by increased mitotic activity in the cells of the uterine glands was found in guinea-pigs of groups II and IV but was not dose related. This lesion was not found in control females.

Skeletal muscle. Degenerative changes in skeletal muscle were most severe and occurred most consistently in guinea-pigs of groups V and VI. The tongue, psoas muscle and rectus femoris muscles had lesions, but the diaphragm was more consistently affected. Affected muscle fibres were swollen, stained darkly with haematoxylin and eosin and often contained calcific granules. In individual fibres, floccular degeneration and dissolution of sarcoplasm were observed. Mineralization of individual fibres (Fig. 6) was most prominent in the muscles of guinea-pigs in groups V and VI. Regeneration was not common.

Bone marrow. Depletion of erythroid cells, with apparent sparing of myeloid cells, was found consistently in guinea-pigs of groups V and VI. In four guinea-pigs, the marrow had scattered necrotic cells.

Lymphoid tissue. Lymphoid necrosis, seen as nuclear pyknosis, karyorrhexis and cytolysis of scattered individual cells, was infrequently found in the mandibular and mesenteric lymph nodes, spleen and thymus of treated animals in all dosage groups. The incidence and severity were not dose dependent.

Lung. Perivascular lymphoid aggregates, bronchopneumonia and inhalation pneumonia were found in animals of all dosage groups and in solvent-dosed controls. The incidence and severity of these pulmonary lesions were similar for treated and control guinea-pigs.

Other tissues. Lesions were seen in the heart and salivary glands of both treated and control guinea-pigs. Cardiac lesions of rhabdomyomatosis were discrete foci of distended and vacuolated muscle fibres often containing centrally located, compact aggregates of striated myofibrils. Disseminated foci of mononuclear inflammatory cells were occasionally seen in the salivary glands. These foci were usually associated with the presence of large, homogeneously staining, intranuclear inclusions in the lining cells of the ductular epithelium, and the finding was consistent with guinea-pig cytomegalovirus infection.

Sections of eye, thyroids, parathyroid, oesophagus, aorta, trachea, adrenal, pancreas, gall bladder, testes, ovaries, vagina, urinary bladder and seminal vesicles were considered histologically normal.

Heat stability of citrinin in solution

Heating citrinin solutions to 83°C for 15 min before administration to male or female guinea-pigs markedly reduced their toxicity. Five males and five females (group V-A) given the heated solution in daily doses of 45 mg/kg all survived the 14-day dosage period, in contrast to average survival times of 7 and 6 days, respectively, for the ten males and ten females given an unheated solution containing the same dose of citrinin. The average percentage weight change in the guinea-pigs given the heated solution was $+15{\cdot}7\%$, while for guinea-pigs given the unheated solution it averaged $-16{\cdot}1\%$. Gross and histopathological alterations were few and mild in the animals given the heated solution, but lesions were frequent and severe in those given the unheated citrinin solution.

DISCUSSION

Clinical features of citrinin toxicity in guinea-pigs, particularly in those given daily intragastric doses of 45 or 50 mg/kg, were weight loss, reduction in spontaneous activity, listlessness, piloerection and unkempt pelage. These signs of toxicosis were not observed consistently, however, and some guinea-pigs were found dead without premonitory signs. The most consistent sign of toxicosis was loss of body weight. Only occasionally were the parasympathomi-

metic effects reported in other species (Ambrose & DeEds, 1946; Carlton *et al.* 1974) seen in our guinea-pigs.

The survival of the guinea-pigs was dose dependent to some degree, but at doses below the LD_{50} few deaths occurred. Daily weight was the only parameter continuously monitored in this study and the daily percentage weight change correlated with dose. At levels of 25 and 30 mg/kg the initial weight losses were recovered after 8–10 days, indicating that some guinea-pigs became tolerant to citrinin administration, as was previously observed in the rabbit (Ambrose & DeEds, 1946).

The multiple-dose 14-day LD_{50} in guinea-pigs was about 43 mg/kg for citrinin given by daily intragastric administration in DMSO-ethanol solution. This value was not markedly different from the single-dose 14-day LD_{50} of 37 mg/kg previously reported (Ambrose & DeEds, 1946) although in that study a different solvent (sodium hydroxide) and route of administration (sc) were used. Both values were considerably lower than the oral LD_{50} of 112 mg/kg obtained for the mouse (Jordan, Carlton & Sansing, 1977).

Gross lesions induced in guinea-pigs by citrinin were found most consistently in the kidneys, liver and gastro-intestinal tract. Swollen and discoloured kidneys like those seen in our guinea-pigs have also been found in citrinin-treated rats (Krogh *et al.* 1970), swine (Friis *et al.* 1969) and dogs (Carlton *et al.* 1974). Intestinal findings in our guinea-pigs included generally scanty contents, subserosal haemorrhage and faecal contents of a watery, fluid consistency. Ileal intussusception, as seen in dogs given citrinin (Carlton *et al.* 1974; Kitchen, Carlton & Tuite, 1977a), was not a feature of citrinin mycotoxicosis in guinea-pigs.

Haematological values seen in this study were not changed in a pattern indicative of changes dependent on the dose of citrinin. The observations suggested that the combination of citrinin and DMSO could have been responsible for the haematological abnormalities. No haematological changes were observed in the rabbit (Ambrose & DeEds, 1946) or dog (Carlton *et al.* 1974) after citrinin administration, but in the studies on these species the solvent was either sodium hydroxide or ethanol. Either the response of the guinea-pig to citrinin differs from that of the rabbit and dog or the solvent was responsible for some part of the changes observed.

Serum-potassium levels were elevated and indicated renal damage, as did the reduction in urinary pH and proteinuria, but specific gravity and urinary sediments were not changed and there were no increases in urinary levels of glucose, blood, bilirubin, urobilinogen or ketones. No alterations in the urinary and serum enzyme activities were found. The failure to find changes indicative of renal damage in some of these parameters could have been a reflection of having only a terminal sample. Prescott & Ansari (1969) found changes in urinary sediment and specific gravity to be transient in their sequential monitoring studies of mercuric chloride nephrotoxicity in rats.

The major histopathological lesions in our citrinin-treated guinea-pigs were found in the kidneys. Necrosis of tubular epithelial cells was most prominent in the convoluted portion of the proximal tubules; increased dosage levels also produced necrosis in the straight segments of the proximal tubules, in the thick limb of Henle and in the distal tubules. These changes were similar to the citrinin-induced renal lesions of rabbits (Ambrose & DeEds, 1946), rats (Krogh *et al.* 1970) and swine (Friis *et al.* 1969). Species differences apparently exist in citrinin nephropathy, however, as the primary tubular necrosis in dogs involved the distal convoluted tubules and the loop of Henle (Carlton *et al.* 1974; Kitchen, Carlton & Tuite, 1977b). Calcification of necrotic tubular epithelial cells was a frequent finding in our guinea-pigs, but has not been described in other species with citrinin nephropathy. Guinea-pigs do not tolerate an excess of a variety of anions and, in particular, are sensitive to alterations in dietary phosphate (Hogan, House & Regan, 1955). Citrinin may have produced a mineral imbalance in our guinea-pigs or the renal damage induced by citrinin may have resulted in phosphate retention. Either mechanism could have resulted in tissue mineralization.

Mineralized hepatocytes around necrotic foci and biliary hyperplasia were found in guinea-pigs given the highest doses of citrinin. Focal hepatic necrosis was not considered a lesion of citrinin toxicosis as the incidence and severity were similar in citrinin-treated and control animals. The aetiology of the hepatic necrosis was not determined. The calcification of hepatocytes was probably related to the same aetiological factors as the renal tubular mineralization of epithelial cells of the renal tubules. Bile-duct hyperplasia was dose related and considered to be a response to citrinin administration.

Necrosis of mononuclear leucocytes in the lamina propria was the most consistent citrinin-related intestinal lesion observed in our test guinea-pigs and was most frequent and severe in the caecum. Gastro-intestinal lesions of acute mucosal inflammation and mucosal ulceration primarily involving the stomach were not frequent. Mason (1971) reported that large oral doses of DMSO (actual dose levels unspecified) caused haemorrhagic gastro-enteritis in rats and dogs. Guinea-pigs, however, tolerated oral doses of 11 mg/kg without untoward effects. The guinea-pigs of our study received 2·7 mg DMSO/kg and no intestinal lesions were observed in solvent-dosed controls. It seems unlikely, therefore, that DMSO was the cause of the intestinal lesions, and they may be attributed in part to the antibiotic activity of citrinin (Oxford, 1942; Timonin & Rouatt, 1944b) since a number of antibiotics effective against Gram-positive bacteria caused greatly dilated caeca containing liquid faeces in guinea-pigs, although consistent histopathological alterations were not found (Eyssen, DeSomer & Van Diick, 1957). The gut lesions in our guinea-pigs were dose related, suggesting a direct relationship between citrinin administration and their development.

Necrotic cells in Huxley's layer of the hair-root bulbs in the skin of test animals was not accompanied by hair loss. The average cyclic hair growth in guinea-pigs is 16–19 wk (Dawson, 1930), so the 2-wk dosage and observation period of this study was not sufficient for the development of hair loss.

Myopathy of skeletal muscles was especially prominent in guinea-pigs given the highest doses of citrinin.

Telford & Einarson (1971) described similar lesions in the skeletal muscles of guinea-pigs fed vitamin E-deficient diets for 2–3 wk. Whether the myopathy in our test animals was a specific response to citrinin administration or a complication of inadequate nutrition was not established. Lesions in the skeletal muscles of citrinin-dosed animals have not previously been described, but it is not always clear whether skeletal muscle was consistently examined. The mineralization of fibres may have resulted from the same processes responsible for hepatic and renal mineralization.

The perivascular lymphoid aggregates seen in the lungs of several guinea-pigs in these trials were consistent with those described in "normal" guinea-pigs (Thompson, Hunt, Fox & Davis, 1962). The distribution was similar among treated animals and controls and the occurrence appeared to be unrelated to citrinin administration. Bronchopneumonia and inhalation pneumonia were due to dosing procedures.

The changes in cardiac muscle occurred in both test and control guinea-pigs and were identical to those of rhabdomyomatosis, described as an incidental lesion in guinea-pigs (Rooney, 1961; Vink, 1969). The incidence and severity of these lesions in our guinea-pigs were not altered by citrinin administration.

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OCHRATOXIN A MYCOTOXICOSIS IN THE GUINEA-PIG

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Abstract—Ochratoxin A dissolved in dimethylsulphoxide–50% ethanol (1:1, v/v) was administered to male and female guinea-pigs in doses of 5·6, 7·5, 10, 13·3 and 20 mg/kg/day by gastric intubation for 14 days. Clinical, clinico-pathological, gross and histopathological observations were made. The 14-day LD_{50} was 9·1 mg/kg for males and 8·1 mg/kg for females. The dose of 5·6 mg/kg was essentially a no-effect dose. Loss of body weight was the most consistent clinical change observed and was roughly proportional to the dose of the mycotoxin. Alterations in clinico-pathological values included increased total plasma protein, decreased serum potassium and chloride, proteinuria and increased serum and urinary lactic-dehydrogenase activities. Gross lesions were not striking, but included pale discoloration of the kidneys, fluid contents and petechiae in the caecum, gastric atony, and congested and enlarged lymph nodes. Major histological changes occurred in the kidneys, lymphoid tissues and gastro-intestinal tract. Renal alterations were degeneration and necrosis of the tubular epithelium, involving both straight and convoluted portions of the proximal tubules, together with tubular dilatation and hyaline-cast formation. Congestion, necrosis and oedema involved lymphoid tissues such as lymph nodes, spleen, thymus and the submucosal lymphoid tissue of the gut. Gastro–intestinal lesions included mucosal hyperaemia, necrosis of mononuclear cells of the lamina propria, and caecal and gastric mucosal ulceration.

INTRODUCTION

Since the first isolation of ochratoxin A from a strain of *Aspergillus ochraceus* (van der Merwe, Steyn, Fourie, Scott & Theron, 1965), the mycotoxin has been obtained from several species of Aspergillus and Penicillium (Ciegler, 1971; Ciegler, Fennell, Mintzlaff & Leistner, 1972; Hesseltine, Vandegraft, Fennell, Smith & Shotwell, 1972; Scott, van Walbeek, Kennedy & Anyeti, 1972). Octratoxin A has been found in cereal grains in the United States (Shotwell, Hesseltine & Goulden, 1969; Shotwell, Hesseltine, Goulden & Vandegraft, 1970), in Canada (Prior, 1976; Scott, van Walbeek, Harwig & Fenwell, 1970; Scott *et al.* 1972), in Denmark (Krogh, Hald & Pedersen, 1973) and in Sweden (Krogh, Hald, England, Rutquist & Swahn, 1974).

Ochratoxin A is a nephrotoxic mycotoxin which has produced renal tubular damage in chickens (Peckham, Doupnik & Jones, 1971), in dogs (Kitchen, Carlton & Tuite, 1977a,b; Szczech, Carlton & Tuite, 1973a,b), in mice (Galtier, Moré & Bodin, 1974), in pigs (Krogh, Axelsen, Elling, Gyrd-Hansen, Hald, Hyldgaard-Jensen, Larsen, Madsen, Mortensen, Moller, Petersen, Ravnskov, Rostgaard & Aalund, 1974; Szczech, Carlton, Tuite & Caldwell, 1973c), in rats (Munro, Moodie, Middleton, Scott & Grice, 1972; Munro, Moodie, Kuiper-Goodman, Scott & Grice, 1974; Purchase & Theron, 1968) and in rainbow trout (Doster, Sinnhuber & Wales, 1972; Doster, Sinnhuber & Pawlowski, 1974), Experimentally, ochratoxin A has produced teratogenic effects in chick embryos (Gilani, Bancroft & O'Rahily, 1975), in hamsters (Hood, Naughton, & Hayes, 1976), in mice (Hayes, Hood & Lee, 1974), and in rats (Brown, Szczech & Purmalis, 1976; Moré & Galtier, 1974).

Ochratoxicosis has not been studied in guinea-pigs, but aflatoxicosis has occurred as a spontaneous disease (Paget, 1954) and has been induced experimentally in that species (Butler, 1966; Butler & Barnes, 1963; Paterson, Crook, Shand, Lewis & Allcroft, 1962). The guinea-pig has been found useful for the study of other mycotoxins, including trichothecenes (Ueno, Ueno, Iitoi, Tsunoda, Enomoto & Ohtsubo, 1971), stachybotryotoxins (Forgacs, 1971) and sporidesmin (Perrin, 1957) and appears to be a suitable species for the investigation of mycotoxic diseases. This report describes the clinical, clinico-pathological and pathological features of ochratoxin A toxicosis in young guinea-pigs.

EXPERIMENTAL

Animals. The distribution of the 43 male and 38 female ochratoxin-treated guinea-pigs among the six test groups is shown in Table 1. In addition, 11 males

Table 1. *Experimental groups given ochratoxin A by gastric intubation*

Group	Dose (mg/kg/day)	No. of guinea-pigs/group	
		Males	Females
I	5·6	5	5
II	7·5	10	10
III	10*	6	—
IV	10	18	11
V	13·3	5	5
VI	20	4	6

*Administered for 7 days only, treatment of the other groups being continued for 14 days.

and nine females were used as solvent-dosed controls. The guinea-pigs, derived from the Hartley strain, were from the colony of the Purdue University School of Veterinary Medicine and were 3–7 wk old and weighed between 250 and 460 g when assigned to the experimental groups. The animals were housed in an air-conditioned building with environmental controls. Fresh drinking-water and commercial guinea-pig diet were provided *ad lib.* Fresh lettuce from a local supermarket was given once weekly. The guinea-pigs were weighed and dosed by gastric intubation each day and were observed several times daily during the experimental period.

Solution preparation and administration. Ochratoxin, A, obtained from a commercial source (Makor Chemicals Ltd, Jerusalem, Israel), was dissolved in dimethylsulphoxide (DMSO)–50% ethanol (1:1, v/v) in appropriate concentrations for administration of the desired dose of mycotoxin in a volume of 5 ml solution/kg body weight. Dosages were calculated daily, and solutions were prepared every third day during the trials and stored in the dark at room temperature. Male and female guinea-pigs were given ochratoxin A doses of 5·6, 7·5, 10·0, 13·3 or 20·0 mg/kg/day for 14 days (Table 1). An additional group of males given 10 mg/kg/day for 7 days was not included in the LD_{50} determination.

LD_{50} determination. This was based on the number of deaths occurring within the 14-day dosing period. The LD_{50} was calculated by the moving average interpolation method of Weil (1952), the constants being as follows: $n = 5$, $k + 1 = 4$ and $d = 0{\cdot}1239$, where n = the number of animals dosed per treatment, $k + 1$ = the number of dosage levels used (the dose levels being 5·6, 7·5, 10 and 13·3 mg/kg in this case) and $d = \log R$, R being the geometric factor used for the difference between successive dose levels and equalling 1·33 in this study.

Clinical pathology. At autopsy blood was collected by cardiac puncture from animals in the groups given doses of 5·6, 7·5, 10 or 20 mg/kg/day (groups I, II, IV and VI respectively), for determinations of packed cell volume by a standard microhaematocrit method, of total concentrations of plasma protein by the refractometer method and of haemoglobin concentrations by a photo-electric reading system (Coulter Hemoglobinometer, Coulter Electronics, Inc., Hialeah, Fla), and for counts of total erythrocytes and leucocytes by an electronic counting device (Coulter Counter, Model Fn, Coulter Electronics, Inc.). Serum concentrations of sodium and potassium ions were determined by flame photometry, and of chloride ion by the method of Cotlove, Trantham & Bowman (1958).

Once during the dosage period or on termination of treatment, solvent-dosed control guinea-pigs and animals from groups I, II, IV and VI were housed overnight in individual mesh-bottom, stainless-steel metabolism cages and urine was collected for determinations of pH, specific gravity, protein, glucose, blood, bilirubin, urobilinogen and ketones (Multistix, Ames Co., Elkhart, Ind.). Urine sediments were stained with Wright's stain for microscopic examination. Portions of urine samples were dialysed for 2 hr in running tap-water for enzyme-activity determinations.

The activities of lactic dehydrogenase (LDH), isocitric dehydrogenase (ICDH), glutamic–oxalacetic transaminase (GOT) and glutamic–pyruvic transaminase (GPT) were determined in serum and urine samples from male and female guinea-pigs using commercially available enzyme test systems (Boehringer Mannheim Corp., New York).

Pathology. Guinea-pigs found dead, those killed when moribund and the survivors killed at the termination of the trials were autopsied. At autopsy, representative portions of most organs were fixed in 10% neutral buffered formalin, processed for paraffin sectioning and stained with haematoxylin and eosin for histopathological examination. Portions of the liver and kidneys from selected test animals and controls were fixed in formol–alcohol and processed by Best's carmine method for glycogen. Selected sections were also stained by Masson's trichrome method or by the periodic acid–Schiff (PAS) method.

RESULTS

Clinical observations

Loss of body weight was the most consistent clinical change seen in guinea-pigs with experimental ochratoxicosis (Figs 1 & 2). Animals given 5·6 mg ochratoxin A/kg/day had weight gains similar to those of the solvent-dosed controls. The loss of body weight in animals of the other groups was roughly proportional to the dose of the mycotoxin given. Other signs of toxicosis, less frequently observed, included a dishevelled pelage and decreased spontaneous activity a day or two before guinea-pigs were found dead or moribund. In most cases, however, impending death was not preceded by signs. Oliguria was seen frequently in guinea-pigs given one of the three highest dose levels of ochratoxin A.

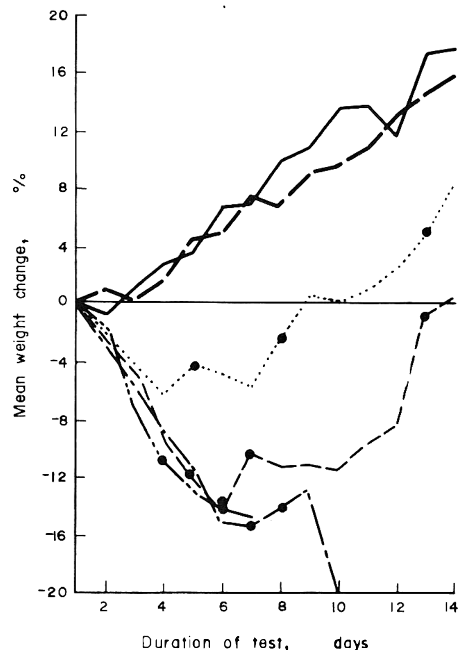


Fig. 1. Mean body-weight changes in male guinea-pigs given 0 (——), 5·6 (——), 7·5 (·····), 10 (——), 13·3 (-—-) and 20 (—--) mg ochratoxin A/kg/day. Times at which deaths occurred are indicated (●).

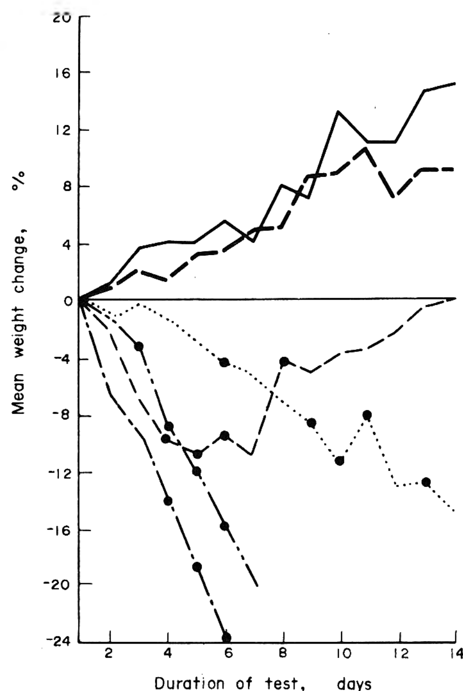


Fig. 2. Mean body-weight changes in female guinea-pigs given 0 (——), 5·6 (— —), 7·5 (· · · · ·), 10 (— —), 13·1 (-—-) and 20 (—--) mg ochratoxin A/kg/day. Times at which deaths occurred are indicated (●).

LD_{50} determinations

The average survival times of guinea-pigs given ochratoxin A were inversely proportional to the dose of mycotoxin. Most of the test animals (17 males and 25 females) died on days 3–14 of the dosing period. The mean survival time was 6·5 days (SD 2·6) in males and 6·2 days (SD 3·0) in females. The 14-day LD_{50} of ochratoxin A given daily by intragastric intubation was 9·1 (95% confidence interval 7·6–10·9) mg/kg/day for males and 8·1 (6·7–9·9) mg/kg/day for females.

Clinico-pathological observations

Haematology. All the test animals showed decreased leucocyte counts (Table 2) and increases in levels of total plasma proteins were especially prominent in animals given 10 or 20 mg ochratoxin/kg/day. Guinea-pigs of all test groups had decreased concentrations of serum Cl^- and K^+, but serum Na^+ levels were not significantly altered (Table 2). Differential leucocyte counts determined for two animals in group II and four animals in group IV were within the normal range.

Urine analysis. Proteinuria was found in guinea-pigs given 10 or 20 mg/kg (Table 3). Oliguria was frequently noted, but anuria occurred only occasionally. The reduced volume was not accompanied by significant changes in the specific gravity. Occasional leucocytes, moderate numbers of semi-clear globules and an abundance of sand-like crystals were in the urinary sediments of both treated animals and controls.

Enzyme activities. Increased LDH activity was found in the urine of guinea-pigs given 10 mg/kg, but urinary activities of GPT, GOT and ICDH did not differ in any group from the control values (Table 3). Serum activities of LDH and GOT were increased in all groups tested except that given the 20-mg/kg doses, in which only LDH was increased. The serum activity of ICDH was increased in guinea-pigs given 20 mg/kg.

Autopsy observations

Gross lesions were observed with only moderate frequency in guinea-pigs given ochratoxin A. Renal changes included generalized paleness or pale mottled discoloration, but the kidneys did not appear swollen or enlarged. Hepatic changes, seen less often, were a general paleness, pale mottling or the presence of multiple white foci. Hyperaemia and mucosal haemorrhage were observed in the uterus.

Gastro-intestinal lesions were most frequent in animals given 7·5 or 10 mg ochratoxin A/kg/day. Gut contents were often reduced and subserosal petechiae were seen occasionally in the caecum. Animals given 10 or 13·3 mg/kg/day had atonic stomachs distended either with gas, fluid or ingesta. Such stomachs often ruptured easily during routine manipulations at autopsy. The serosa was mottled grey and the mucosa was grey-black and covered with thick dark mucus.

Table 2. *Haematological and serum-electrolyte data for guinea-pigs given ochratoxin A in daily doses of 5·6–20 mg/kg*

Parameter		Mean values ± 1 SD for group				
		O	I	II	IV	VI
	Dose (mg/kg/day)...	0	5·6	7·5	10	20
	No. of animals sampled...	15	10	2	14	3
PCV (%)		38 ± 4	32 ± 3	37 ± 4	35 ± 7	33 ± 3
TPP (mg/100 ml serum)		4·9 ± 0·3	4·5 ± 0·3	8·6 ± 4	5·2 ± 0·7	7·9 ± 3·0
Hb (mg/100 ml serum)		14·3 ± 1·6	11·8 ± 1·6	12·8 ± 0·8	12·7 ± 1·1	13·1 ± 1·3
RBC (10^6/mm^3)		5·4 ± 0·7	4·0 ± 1·4	5·3 ± 0·4	5·0 ± 0·5	4·8 ± 1·2
WBC (10^3/mm^3)		15 ± 22	2 ± 0·9	3 ± 0·5	9 ± 15	5 ± 4
Na^+ (mequiv/litre)		116 ± 30	129 ± 26	132 ± 5	114 ± 25	114 ± 15
K^+ (mequiv/litre)		7·7 ± 3·5	6·2 ± 0·9	5·9 ± 0·5	5·9 ± 1·2	6·0 ± 1·0
Cl^- (mequiv/litre)		103 ± 8	91 ± 3·2	91 ± 4	93 ± 2·3	92 ± 3·1

PCV = Packed cell volume TPP = Total plasma protein Hb = Haemoglobin RBC = Erythrocytes
WBC = Total leucocytes

Table 3. *Urine analysis and enzyme activities of guinea-pigs given ochratoxin A in daily doses of 5·6–20 mg/kg*

Parameter		Mean values ± 1 SD for group				
		0	I	II	IV	VI
	Dose (mg/kg/day)...	0	5·6	7·5	10	20
	No. of animals sampled...	15	10	2	14	3
Urine*						
pH		8·3 ± 0·8	8·8 ± 0·2	8·4 ± 0·7	8·1 ± 1·4	8·2 ± 0·7
Specific gravity		1·020 ± 0·010	1·026 ± 0·008	1·020 ± 0·004	1·018 ± 0·009	1·026 ± 0·010
Protein		0·5 ± 0·8	0·6 ± 0·25	0·5 ± 0·2	1·7 ± 1·2	1·8 ± 0·5
Enzymes (IU/litre)						
LDH		57 ± 108	11·1 ± 8·4	54 ± 72	107 ± 137	12·5 ± 14
GOT		4·9 ± 4·2	3·5 ± 3·3	28 ± 12	41 ± 78	6·4 ± 2·5
GPT		3·8 ± 4·5	4·6 ± 1·7	4·6 ± 3·2	16 ± 14	ND
ICDH		12·8 ± 12·5	ND	16 ± 14·2	17 ± 16	5·6 ± 6·3
Serum						
Enzymes (IU/litre)						
LDH		296 ± 199	680 ± 250	694 ± 220	773 ± 452	489 ± 370
GOT		53 ± 30	110 ± 27	94 ± 33	81 ± 47	45 ± 9·6
GPT		24 ± 32	18 ± 4	22 ± 11	9·2 ± 2·7	ND
ICDH		52 ± 16	ND	ND	60 ± 75	108 ± 154

LDH = Lactic dehydrogenase GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase
ICDH = Isocitric dehydrogenase ND = Not determined
*Urinary sediment examinations and tests for glucose, blood, bilirubin, urobilinogen and ketones showed no changes.

Caecal contents were occasionally of a fluid consistency.

Lymph nodes of the head, neck and abdomen were infrequently enlarged and congested. The thymuses of a few guinea-pigs were small and hyperaemic. No gross lesions were observed in control guinea-pigs or in those given 10 mg/kg/day and killed after 7 days.

Histopathology

The major histopathological alterations in guinea-pigs with experimental ochratoxicosis were in the kidneys, lymphoid tissue and gastro-intestinal tract.

Kidney. The kidneys showed changes in nearly all test animals except those given 5·6 mg ochratoxin A/kg/day, but renal damage was minimal to slight in the animals given the lower doses. Degeneration and necrosis were most severe and frequent in the epithelial cells of the straight segments of the proximal tubules (Fig. 3). Nuclear pyknosis, karyorrhexis and cytoplasmic vacuolation of the renal epithelial cells generally involved either single cells or small groups primarily in the convoluted and straight segments of the proximal tubules, but in areas of severe tubular degeneration, nuclear karyorrhexis and cytoplasmic vacuolation involved nearly all epithelial cells. Loss of the brush border was most evident in sections stained with PAS or Masson's trichrome method. Groups of large pleomorphic, immature and often multinucleate tubular epithelial cells were found, but these were not common (Fig. 4). No calcification or desquamation of degenerated tubular epithelial cells was found. Lesions in other regions of the nephrons were infrequent in guinea-pigs given the lower doses. Hyaline casts, present at various levels of the nephron tubules, resembled tubules filled with proteinaceous fluid, except that the casts were more compact and were often outlined because of contraction from the tubular wall (Fig. 3). Tubular dilatation was occasionally present and was often associated with hyaline casts. Mitotic activity was increased in the tubular epithelial cells of guinea-pigs given the lower doses and was most prominent in guinea-pigs given 10 mg/kg/day and killed after 7 days of dosing.

Hyaline-cast formation and tubular dilatation became more common and extensive as the dosage of ochratoxin A was increased, except for the guinea-pigs given 20 mg/kg/day. The changes were probably less severe in this group because the survival times were much shortened.

Lymphoid tissue. Congestion, necrosis and mild oedema were present in the mandibular and mesenteric lymph nodes, spleen, thymus and submucosal lymphoid tissue of the gastro-intestinal tract (Fig. 5). The incidence of these changes was related to dose and they were most prominent, but not extensive, in guinea-pigs given 13·3 or 20 mg ochratoxin A/kg/day. Necrosis of the lymph nodes was characterized by scattered individual cells or groups of cells with pyknotic or karyorrhectic nuclei. Necrotic foci were haphazardly dispersed throughout the nodes and were found in germinal centres, perifollicular areas and medullary rays. Oedema of the lymph nodes was usually mild to moderate in degree. Congestion of trabecular vessels and cortical capillaries was often prominent.

Necrosis was frequently present in the spleen, but it was not extensive and was seen primarily in females, in which the occurrence was dose related. Nuclear pyknosis or karyorrhexis involved scattered individual lymphocytes of the splenic corpuscles. Necrosis of widely scattered individual lymphocytes occurred in the thymus, involving mainly the cells of the cortex. The thymic vasculature was often moderately congested. No alterations were seen in the thymic medulla.

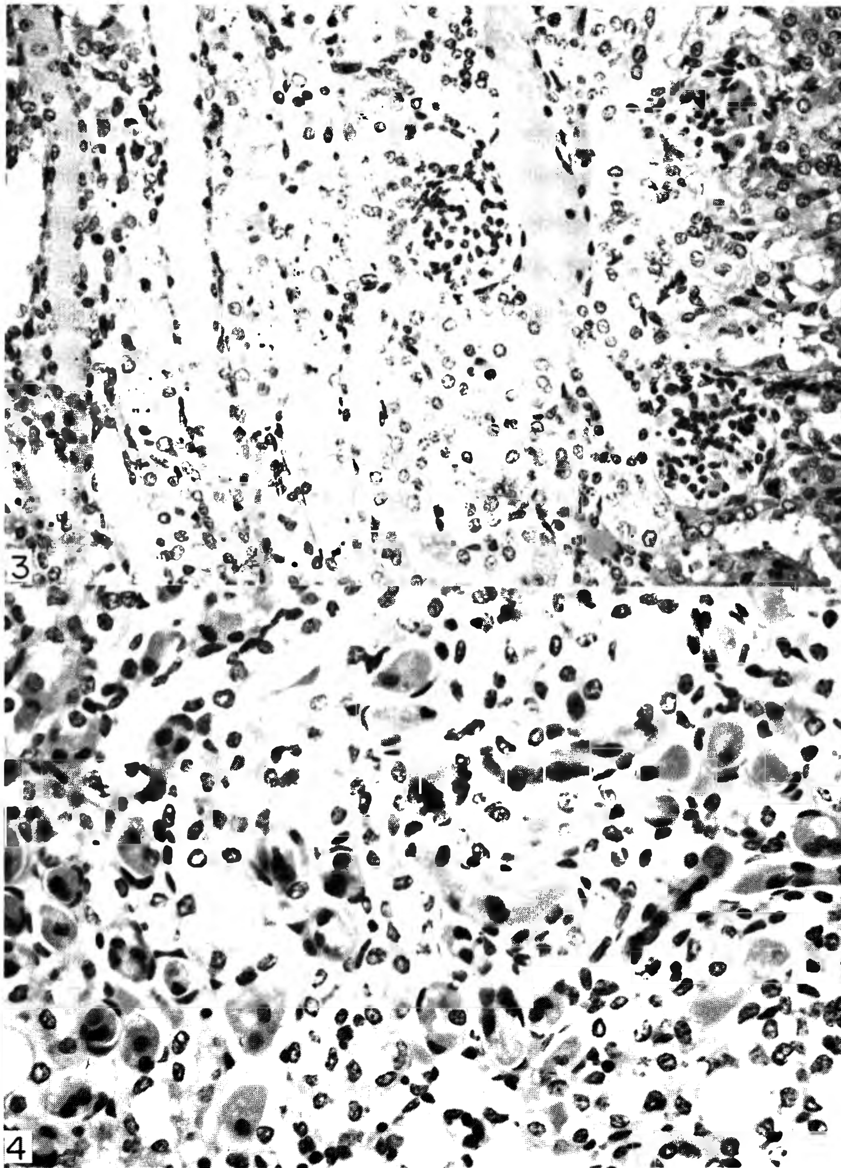
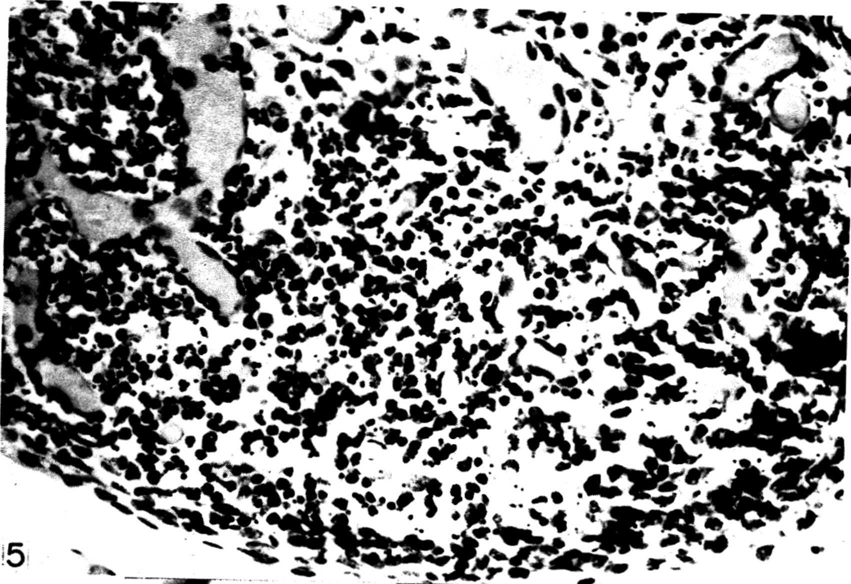


Fig. 3. Necrotic proximal tubules and hyaline casts in the kidney of a guinea-pig given ochratoxin A. Haematoxylin and eosin × 224.

Fig. 4. Pleomorphic tubular epithelium in the renal medulla of a guinea-pig given ochratoxin A. Haematoxylin and eosin × 350.



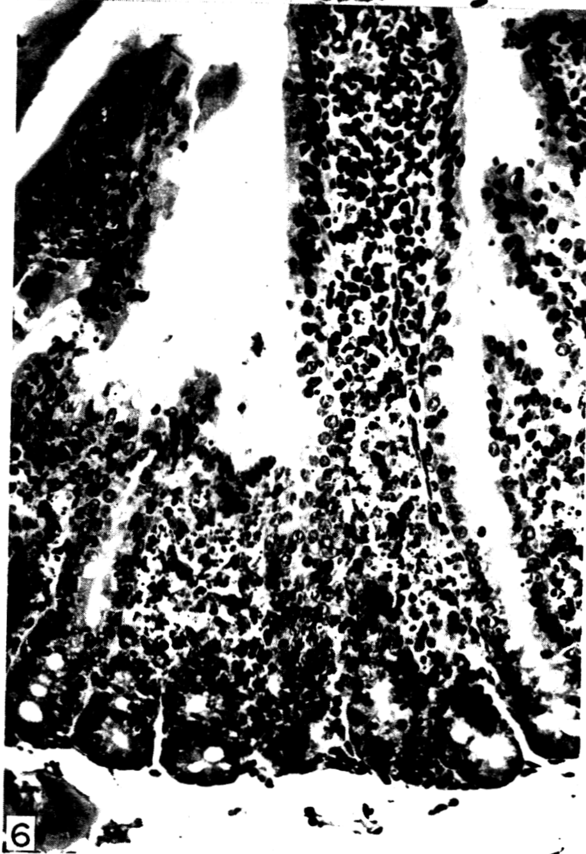
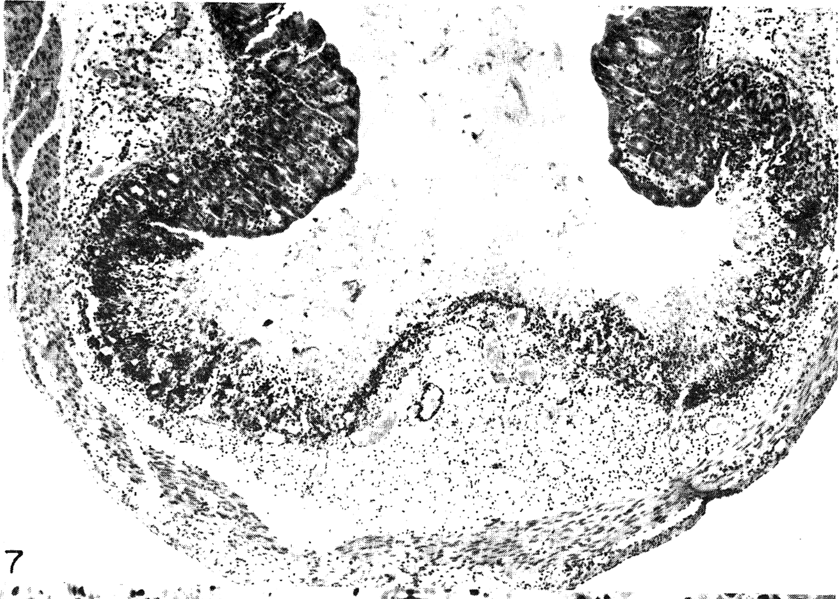


Fig. 5. Congestion and necrosis in the mandibular lymph node of a guinea-pig given ochratoxin A. Haematoxylin and eosin ×224.

Fig. 6. Necrosis of mononuclear leucocytes in the lamina propria of the duodenum of a guinea-pig given ochratoxin A. Haematoxylin and eosin ×224.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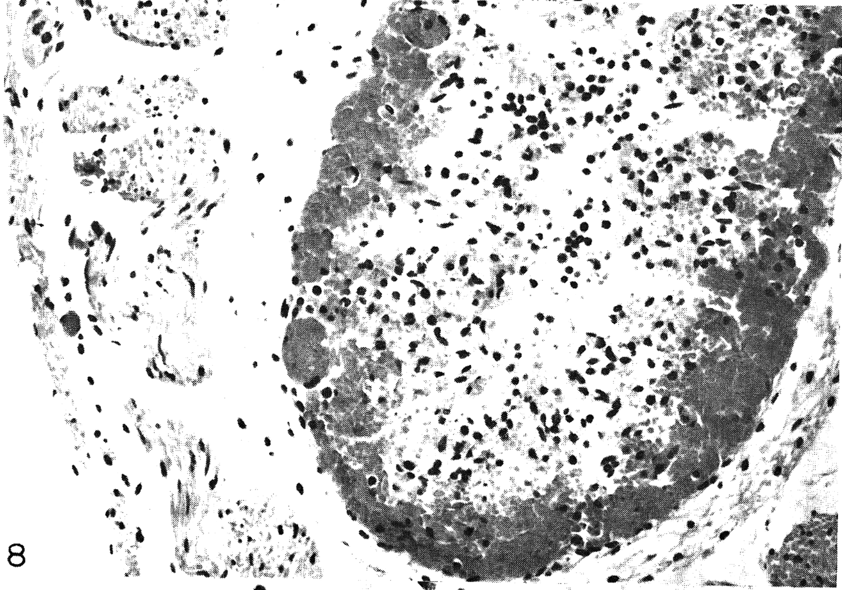
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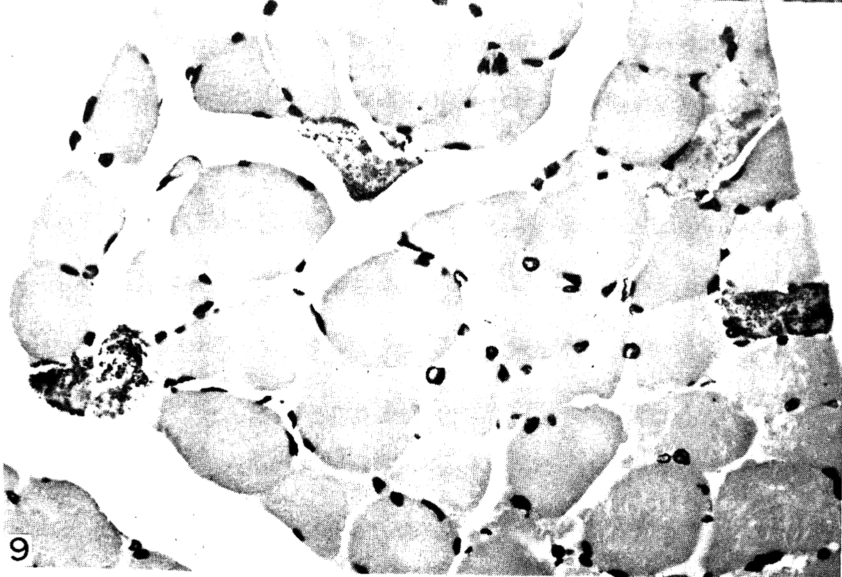
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Fig. 7. Necrosis and hyperaemia of the caecal mucosa of a guinea-pig given ochratoxin A. Haematoxylin and eosin ×224.

Fig. 8. Ulceration of the gastric mucosa with submucosal congestion and oedema in a guinea-pig given ochratoxin A. Haematoxylin and eosin ×56.

Fig. 9. Swelling and mineralization of fibres of the rectus femoris muscle in a guinea-pig given ochratoxin A. Haematoxylin and eosin ×350.

Necrosis of the submucosal lymphoid tissue of the gastro-intestinal tract was usually mild and involved scattered individual lymphocytes, as in other sites. Abscess formation was observed in the submucosal lymphoid tissue but was uncommon. Few or no alterations occurred in the overlying intestinal mucosa.

Intestine. Hyperaemia of the mucosal and submucosal vessels and necrosis of mononuclear leucocytes of the lamina propria were observed consistently (Fig. 6). Necrosis of leucocytes of the lamina propria and vascular congestion were frequently seen in the duodenum, jejunum, ileum and colon. Necrotic cells were usually located below an intact and normal intestinal epithelium. Caecal lesions were most frequent and severe in guinea-pigs given the 13·3 or 20 mg/kg/day doses. Ulcerated areas found in three guinea-pigs were not associated with necrosis in the underlying Peyer's patches or small lymphoid aggregates (Fig. 7). Intestinal lesions were not found in control animals.

Stomach. Gastric lesions consisted of congestion of mucosal vessels, mild submucosal oedema and occasional mucosal ulceration (Fig. 8). The incidence and severity correlated somewhat with the dose of ochratoxin A, but the gastric lesions were neither as common nor as severe as the intestinal lesions.

Skin. Nuclear pyknosis, karyorrhexis and cytoplasmic vacuolation involved the mononuclear cells of Huxley's layer of the hair-root bulbs. These changes, more common and severe in the higher dose groups, were prominent in guinea-pigs given 13·3 mg/kg and were not found in the skin of control guinea-pigs. Mitotic activity was increased in cells of Huxley's layer in the treated animals. No significant histopathological alterations were found in adjacent adnexal structures, subcutis, dermis or epidermis.

Uterus. The vascular layer of the endometrium was consistently congested in females given high doses of ochratoxin A. Oedema of the myometrium and endometrium was observed occasionally. Uterine congestion was generally dose related.

Liver. Hepatic lesions consisted of focal necrosis, biliary hyperplasia and calcification of hepatocytes. Biliary hyperplasia, when present, was disseminated, but never extensive, and its incidence generally increased with increasing doses of ochratoxin A. Focal hepatic necrosis occurred sporadically in most treatment groups but it was not dose related and it occurred also in control females. Mineralization involved either single cells or focal aggregates of hepatocytes. It showed no correlation with increasing ochratoxin A dosage but was not found in control guinea-pigs.

Centrilobular fatty change and the presence of glycogen occurred occasionally in hepatocytes of test and control animals. No relationship with ochratoxin A administration was demonstrated.

Skeletal muscle. Degenerative changes, seen occasionally in tongue muscle, psoas muscle, diaphragm and rectus femoris muscle, included swollen fibres, floccular degeneration of sarcoplasm and sarcoplasmic accumulation of minute calcium particles (Fig. 9). Lesions of skeletal muscle, while never extensive, were most common and severe in guinea-pigs given 7·5 mg/kg/day. These lesions were not seen in control guinea-pigs.

Lung. Pulmonary changes included perivascular lymphoid aggregations, bronchopneumonia and inhalation pneumonia and their incidence and severity were similar in treated and control guinea-pigs.

Cardiac muscle. Occasionally ochratoxin A-treated animals and solvent-dosed controls showed variably sized, discrete foci of distended cardiac muscle fibres. These had centrally located compact aggregates of striated myofibrils within clear sarcoplasmic vacuoles. Cardiac muscle of any depth could be involved, but the most frequently involved region was the subendocardium. Normal myocardial fibres, endocardium or epicardium were found adjacent to and surrounding the lesions.

Other tissues. Sections of eye, thyroid, parathyroid, oesophagus, aorta, trachea, adrenal, pancreas, gall bladder, testes, ovaries, vagina, urinary bladder, seminal vesicles and bone marrow were considered histologically normal.

DISCUSSION

The most consistent clinical feature of ochratoxicosis A in guinea-pigs was loss of body weight, with only occasional animals showing decreased spontaneous activity and a dishevelled pelage. Reasons for the weight loss were not established, as feed and water intake were not recorded, but increases in total serum protein and the appearance of the carcass at autopsy indicated some degree of dehydration, and it appeared that dehydration because of decreased water consumption was at least partly responsible for the weight loss.

The dose of 5·6 mg ochratoxin A/kg/day produced no clinical signs of toxicity, few histological lesions and no significant alteration in weight gains, suggesting that this was close to a no-effect dose. Increased doses of ochratoxin A produced proportional reductions in body-weight gains and in mean survival times. Guinea-pigs given either 7·5 or 10 mg ochratoxin A/kg daily had lost between 6 and 14% of their initial body weight by days 6-7 of the trial. Some guinea-pigs recovered their weight loss and, in a few, the body weight increased above the starting weight, indicating development of some tolerance to the mycotoxin even during continued dosing. Tolerance or an increased resistance to the renal injury produced by ochratoxin A was evidenced by the increased reparative activity in the kidneys, prominent especially in guinea-pigs given 10 mg/kg and killed after 7 days of dosing. Other authors (Harrison & Harrison, 1954; Kruś & Zaleska-Rutczyńska, 1972;

Prescott & Ansari, 1969) have reported the development of tolerance to such nephrotoxic agents as mercuric chloride, maleic acid and chloroform during periods of continued toxin administration. Balazs (1974) found that repeated administration of mercuric chloride, maleic acid, salicylic acid, chloroform, ozone, nitrogen dioxide, phosgene or chloropromazine was followed by the development of tolerance in the target tissues.

The 14-day LD_{50} of ochratoxin A dissolved in DMSO–ethanol and given by intragastric administration was 9·1 mg/kg/day for males and 8·1 mg/kg/day for females. These values were lower than those obtained in most rodent studies. Of the species studied so far, young dogs were the most sensitive (Kitchen, Carlton & Tuite, 1977a,b; Szczech, Carlton & Tuite, 1973a,b) and mice were the most resistant (Galtier, Moré & Bodin, 1974). Because of the development of tolerance, the single-dose LD_{50} may be similar to the daily multiple-dose LD_{50}. However, the single-dose LD_{50} was not determined in this study.

Leucopenia developed in some treated guinea-pigs, but the response was not dose related and the groups given the lowest dosage (5·6 mg/kg/day) had the lowest of the monitored leucocyte counts. Leucopenia in some guinea-pigs may have reflected the lymphoid necrosis present in several lymphoid tissues, but, in others there was no lymphoid necrosis and the cause of the leucopenia was not evident. It may have been merely an example of the variation known to occur in leucocyte counts in guinea-pigs under controlled experimental conditions (Zeman & Wilber, 1965) rather than a response to ochratoxin A administration.

Concentrations of K^+ and Cl^- in the sera of test guinea-pigs were consistently lower than those of controls, but serum Na^+ levels remained relatively unchanged. These changes in serum electrolytes probably reflected the renal damage caused by ochratoxin A. Pitts (1974) reported on the relationship between K^+ secretion and acid-base balance, pointing out that K^+ secretion in the urine was depressed by acidosis and enhanced by alkalosis. The urine of the guinea-pigs in this study was consistently alkaline and serum chloride levels were reduced below the normal range (an indication of alkalosis). It would be expected that K^+ concentrations in the urine would be increased and serum levels decreased. These findings can be contrasted with those found in guinea-pigs given citrinin; in these acidoses was evidenced by the relative acidity of the urine, normal serum Cl^-, proteinuria and increased serum K^+ levels (Thacker, Carlton & Sansing, 1977). The difference in response produced by the two mycotoxins may reflect either the acidity of the citrinin molecule or damage at different levels of the nephron or both factors.

Urine analysis and enzyme determinations provided evidence that ochratoxin A produced a primary toxic effect on the renal tubular epithelium in guinea-pigs, as in other species (Doster *et al.* 1974; Peckham *et al.* 1971; Purchase & Theron, 1968; Szczech *et al.* 1973a,b). The activity of LDH in the urine was increased. Activities of enzymes in the urine apparently originate from renal tubular epithelial cells, as the molecular weight of LDH (130,000), for example, is in excess of the upper molecular-weight limit for proteins found to pass into the glomerular filtrate (Raab, 1972). Failure to observe epithelial desquamation and alteration of urinary specific gravity may have been due to the lack of continuous monitoring of the urine, as Prescot & Ansari (1969) found these changes to be transient in mercuric chloride nephrotoxicity in rats.

Necrosis of the renal tubular epithelium mainly involved the convoluted and straight segments of the proximal tubules in our guinea-pigs. Renal lesions were dose related and survivors frequently showed histological evidence of renal tubular regeneration, including mitotic activity and foci of large pleomorphic and often multinucleate epithelial cells. Cells of similar appearance were observed in rats with ochratoxicosis (Munro *et al.* 1974).

Necrosis of lymphoid cells, found in the mandibular and mesenteric lymph nodes, spleen, thymus and submucosal lymphoid aggregates of the gastro-intestinal tract, was frequent but never extensive. Lymphoid necrosis has been produced in guinea-pigs by members of the 12,13-epoxytrichothecene group (Tatsuno, Saito, Enomoto & Tsunoda, 1968) and by stachybotryotoxin (Forgacs, 1971) and is thus not a lesion specific to ochratoxicosis A.

Gastro-intestinal lesions in the guinea-pigs were not as severe as those that have been described in chicks (Peckham *et al.* 1971), dogs (Kitchen *et al.* 1977a,b; Szczech *et al.* 1973a,b), swine (Szczech *et al.* 1973c) and rats (Galtier *et al.* 1974; Purchase & Theron, 1968). In guinea-pigs, necrotic mononuclear leucocytes occurred in the lamina propria of all segments of the intestinal tract, but necrotic cells were most numerous in the caecal mucosa. Mucosal ulceration was seen occasionally in the stomach and caecum of guinea-pigs given the highest doses. In contrast to dogs (Szczech *et al.*, 1973b) ulceration was seldom found overlying necrotic lymphoid aggregates.

Necrosis and cytoplasmic vacuolation were seen in the cells of Huxley's layer of the hair-root bulbs. The incidence and severity were related to the ochratoxin A dose. The skin of the guinea-pig is sensitive to other mycotoxins, including trichothecenes applied topically (Gláz, Scheiber, Gyimesi, Horvath, Steczek, Szëntirmai & Bohus, 1959; Gláz, Scheiber & Járfás, 1960). Parenteral or oral administration of trichothecenes (Tatsuno *et al.* 1968; Ueno *et al.* 1971) caused necrosis of the gastro-intestinal mucosa and lymphoid necrosis, but not lesions of the skin.

Skeletal muscle degeneration was observed in ochratoxin A-treated guinea-pigs, the distribution in the tongue muscle, psoas muscle, diaphragm and rectus femoris muscle being similar to that in guinea-pigs given citrinin (Thacker *et al.* 1977). Individual fibres were swollen and showed floccular degeneration and accumulation of calcium in the sarcoplasm. Such lesions of skeletal muscle have not been described previously in a mycotoxicosis. They were unlike those of a spontaneous myositis of guinea-pigs (Saunders, 1958) or the syndrome produced by diets excessive in phosphorus (Hogan, House & Regan, 1955) but were similar to the myopathy of vitamin E deficiency (Telford & Einarson, 1971). Whether vitamin E deficiency was a factor in the development of the myopathy in the guinea-pigs given ochratoxin A was not determined.

Spontaneous alterations found in both test and control guinea-pigs and not modified by ochratoxin A administration included perivascular lymphoid hyperplasia in the lungs (Thompson, Hunt, Fox & Davis, 1962) and cardiac rhabdomyomatosis (Rooney, 1961; Vink, 1969).

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ACUTE ORAL TOXICITY OF ETHYLIDENE GYROMITRIN IN RABBITS, RATS AND CHICKENS

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Abstract—The acute oral toxicity of ethylidene gyromitrin (acetaldehyde *N*-methyl-*N*-formylhydrazone), the toxic compound in false morels, *Gyromitra esculenta*, was determined in rabbits, rats and chickens, the LD_{50} values (with 95% confidence limits in parentheses) in these three species being 70 (67–74), 320 (210–494) and >400 mg/kg, respectively. Clonic–tonic convulsions, hypersensitivity, loss of activity, lack of appetite and severe weight loss accompanied intoxication in rabbits and rats. Haemoglobinuria, proteinuria, bilirubinuria and a decrease in urinary pH were evident in affected rabbits and concentrations of creatine, bilirubin and activities of aspartate and alanine aminotransferases were abnormally high in the serum. Rabbits that died showed extensive fatty degeneration of the liver, but this effect was much less severe in rats. Rabbits excreted at least part of the administered ethylidene gyromitrin unchanged in the urine. No toxic effects were detected in chickens given a dose of 400 mg/kg.

INTRODUCTION

False morels, *Gyromitra esculenta*, are traditionally widely consumed in Finland because of their delicious aroma and flavour. They are also important commercial mushrooms, accounting for about 27% of the canned mushrooms marketed by the main mushroom producer in Finland, the Valio Finnish Cooperative Dairies' Association. In addition, Valio markets these mushrooms in dried form, and also fresh when they are available.

Since false morels are known to possess toxic volatile components, they are traditionally dried or cooked before use. The main toxic compound is ethylidene gyromitrin or acetaldehyde *N*-methyl-*N*-formylhydrazone, the concentration of which is about 1·2–1·6 g/kg in fresh mushrooms obtained from European sources (List & Luft, 1969). Pyysalo (1975) and Pyysalo & Niskanen (1976) have found a total of seven other hydrazones in false morels, in addition to ethylidene gyromitrin. Fresh or inadequately boiled false morels have caused serious poisoning, some cases of which have been fatal. A review by Franke, Freimuth & List (1967) reported that the effects varied from case to case, and pointed out that the doses involved had not been measured either in man or in laboratory animals. The same review reported damage to the eyes, mucous membranes and lungs in people who had inhaled steam from false morels being processed on an industrial scale.

More recently, Schmidlin-Mészáros (1974 & 1975) has calculated lethal doses of ethylidene gyromitrin from the amounts of false morels reported in the literature to cause intoxication, deriving a lethal dose for children of 10–30 mg/kg body weight and for adults of 20–50 mg/kg. Some preliminary experiments on the toxicity of ethylidene gyromitrin in laboratory animals have been carried out by List & Luft (1968) to support chemical analyses, but as yet no determinations of acute oral toxicity or lethality have been made in accordance with WHO standards.

Since false morels are important commercial mushrooms nowadays in Finland, it was necessary to obtain more exact information about the effects and toxic potency of ethylidene gyromitrin. The effects of single doses and the acute oral toxicity of ethylidene gyromitrin have been determined, therefore, in rabbits, rats and chickens. These acute toxicity tests were performed in the Laboratory for Experimental Animals in the Faculty of Agriculture and Forestry of the University of Helsinki during the period 1 March to 31 July, 1975.

EXPERIMENTAL

Material. Ethylidene gyromitrin (98–100% pure; b.p. 143°C; density 1·05 g/cm³) was synthesized by H. Pyysalo according to the method of List & Luft (1968).

Animals. Species, group sizes, numbers of dose levels and the dosage volumes complied with the recommendations of Balazs (1970), the Joint FAO/WHO Expert Committee on Food Additives (1974), Loomis (1974) and the *Pharmacopea Nordica* (1968). The experimental conditions and feeding procedures were those recommended by UFAW (1972). New Zealand White rabbits were divided into a control group of five animals and three groups of six given 65, 70 or 75 mg ethylidene gyromitrin/kg in a single dose. The dose levels (in mg/kg, with numbers/group in parentheses) given to groups of Sprague–Dawley rats were 0 (control; six animals), 200 (four), 250 (eight), 300 (ten), 325 (ten) and 350 (ten). Five groups of six Pirch de Kalb chickens were given 0 (control), 50, 100, 200 or 400 mg/kg.

Clinical and histological studies. Blood and urine samples were taken from the rats and rabbits at intervals throughout the observation period (up to 15 days) and terminally, the urine samples for pH, glucose, protein, blood, bilirubin, phenylketone and ethylidene gyromitrin determinations, the blood samples for magnesium, creatine, bilirubin, aspartate amino-

Table 1. *Incidence and times of death in rabbits and rats given a single dose of ethylidene gyromitrin by intubation*

Dose (mg/kg)	No. of animals treated	No. of deaths	Time of deaths (days after dosing)
		Rabbits	
65	6	1	8·5
70	6	2	3–3·5
75	6	5	0·5–3
		Rats	
200	4	0	—
250	8	1	8
300	10	2	6–8
325	10	3	2–8
350	10	8	2–8

transferase (ASAT), alanine aminotransferase (ALAT) and ornithine carbamoyltransferase (OCT). Urinary ethylidene gyromitrin was determined by H. Pyysalo using glass-capillary gas chromatography and NMR spectroscopy (Pyysalo, 1975). Samples of liver, kidney, spleen, heart, lung and adrenals were taken for histological examination from rats and rabbits that died or were killed.

Statistical evaluation. LD_{50} values were determined according to the recommendations of the Joint FAO/WHO Expert Committee on Food Additives (1974) and analysed statistically by the method of least squares, 95% confidence limits being calculated according to the method of Litchfield & Wilcoxon (1949).

RESULTS

Table 1 shows the incidence and times of death for the rabbits and rats treated with ethylidene gyromitrin. The oral LD_{50} value for rabbits was 70 mg/kg (95% confidence limits 67–74 mg/kg) and that for rats was 320 mg/kg (210–494 mg/kg).

Table 2 summarizes the incidence of the main signs of intoxication in rabbits treated with different dose levels of ethylidene gyromitrin. In the rats, a dose of 200 mg/kg had no adverse effects, but all the other treated animals had convulsions, hypersensitivity and loss of activity and appetite.

Blood was never present in rabbit urine on the day of dosing (day 0) but it usually appeared the day after. Proteinuria was a typical effect of treatment in rabbits, high concentrations (300 mg/100 ml) being found even after recovery. A marked bilirubinuria was recorded in samples taken 3 days after a dose of 70 or 75 mg/kg, but this disappeared during recovery. Similarly the urinary pH decreased after treatment but returned to normal during recovery. Ethylidene gyromitrin (50 ppm) was detected in urine collected 2 days after administration of a dose of 75 mg/kg. In rats, blood and protein appeared in the urine on days 1, 2 and 3 after dosing, but disappeared again within 1 wk.

Blood samples taken from treated rabbits had much higher creatine, bilirubin, ASAT and ALAT values, and slightly higher magnesium and OCT values than the control samples (Table 3). The creatine value, which had risen on day 1, returned to normal after day 2, while bilirubinaemia reached a maximum 2 days after dosing. Bilirubinaemia was the only exceptional feature detected in the blood serum of the treated rats.

The only detected histological change caused by ethylidene gyromitrin was fatty degeneration in the liver. This was extensive in the rabbits that died as a result of treatment, and occurred slightly in half of the rats receiving a dose of 300 mg/kg or more.

No signs of intoxication were observed in the chickens, even at the highest dose level (400 mg/kg).

DISCUSSION

Tolerance to ethylidene gyromitrin differed widely in the rabbits, rats and chickens, with rats tolerating more than four times the dose tolerated by rabbits, and chickens showing no signs of intoxication at a dose level six times higher than the LD_{50} for rabbits. This finding accords well with the results reported by List & Luft (1968) concerning variations in the tolerance of rabbits, rats, mice and guinea-pigs to ethylidene gyromitrin.

The adverse effects were similar in rabbits and rats, although the latter succumbed to or recovered from the toxin much faster. It has been reported (Franke *et al.* 1967) that in human cases of *Gyromitra esculenta* poisoning, acute or subacute degeneration of the liver is usually evident only 24 hr after ingestion, before the onset of convulsions, while the first sign

Table 2. *Incidence of major signs of ethylidene gyromitrin intoxication in rabbits*

Adverse effect	Dose (mg/kg)...	No. of treated animals affected*		
		65	70	75
Mortality		1	2	5
Severe convulsions		5	5	5
Apathy		6	6	3†
Aphagia		4	5	3†
Tachypnoea		1	3	1†
Low rectal temp. 1 day after dosing		0	3	2†

*Number affected out of a total of six treated, except where indicated otherwise.
†Death ensued so rapidly in three of the rabbits given the 75-mg/kg dose that observation of these effects was impossible; the numbers given are therefore those affected out of a total of three.

Table 3. *Results of serum analyses for rabbits given a single dose of ethylidene gyromitrin by intubation*

Serum component		Range of values in		
		Controls	Rabbits given 65 mg EG/kg	
	Time after dosing (days)...	—	2	14–15
	No. of animals...	5	6	5
Creatine (μmol/litre)		71–80	115	49–75
Bilirubin (μmol/litre)		1·2–2·4	4·0–4·8	0·4–2·0
Magnesium (mmol/litre)		0·75–0·85	1·05–1·35	1·10–1·45
ASAT (IU/litre)		18–20	31–49	18–27
ALAT (IU/litre)		20–31	30–43	28–30
OCT (IU/litre)		0·2–0·5	0·5–0·8	0·2–0·3

EG = Ethylidene gyromitrin ASAT = Aspartate aminotransferase
ALAT = Alanine aminotransferase OCT = Ornithine carbamoyltransferase

in the animals in the present investigation was always clonic–tonic convulsions. The occurrence of severe convulsions support the view that ethylidene gyromitrin affects the central nervous system, as has been found to be the case in people who have eaten fresh false morels (Breuer & Stahler, 1966; Franke *et al.* 1967; Stuhlfauth & Jung, 1952/54).

The increase in the activity of the amino transferases (ASAT and ALAT), and especially of liver-specific OCT in the serum of the rabbits, the increase in the bilirubin content of the urine and the extensive fatty degeneration of the liver of the rabbits demonstrate that ethylidene gyromitrin has a major effect on liver tissue. The proteinuria detected in both rabbits and rats and the abnormally high creatine content in the rabbit serum indicate that ethylidene gyromitrin also attacks the kidneys. It must be noted, however, that high creatine values can result from damage to tissues other than the kidney and that the degree of proteinuria is not a measure of kidney damage.

One effect found in both rabbits and rats in the present investigation was haemoglobinuria, a finding that has been reported also in man (Breuer & Stahler, 1966; Franke *et al.* 1967; Gray, 1972; Stuhlfauth & Jung, 1952/54).

According to Gray (1972), ethylidene gyromitrin hydrolyses to monomethylhydrazine, a very toxic compound. Schmidlin-Mészáros (1974) has reported that the toxicity of ethylidene gyromitrin is due in fact to its decomposition to methylhydrazine and other hydrazine derivatives. According to Pyysalo (1975), other hydrazones in false morels also give rise to methylhydrazine. Gray (1972) has reviewed experiments on apes carried out in the US Air Force Laboratory, according to which the difference between the 'no-effect value' and the lethal value of methylhydrazine is small. The results presented here show that at least some of the ethylidene gyromitrin is not degraded to methylhydrazine *in vivo*, because the presence of the unchanged toxin was clearly demonstrated in the urine of rabbits given a dose of 75 mg/kg.

There is only one report on the toxicities of other hydrazones found in false morels. This shows that rabbits given the pentanal, 3-methylbutanal or hexanal *N*-methyl-*N*-formylhydrazone in a dose of 350 mg/kg body weight died within 24 hr (Pyysalo, 1975). On the basis of the time of death and the dose of these hydrazones it appears that ethylidene gyromitrin is the most toxic component of *Gyromitra esculenta*, because, in rabbits, a dose of only 75 mg/kg was fatal within 12 hr.

Although the LD_{50} values found for ethylidene gyromitrin in this investigation suggest that it is not a very strong poison, its danger lies in the great variation in individual tolerances, as indicated both by human case histories (Franke *et al.* 1967) and by the wide confidence limits of the LD_{50} values established for rats in the present investigation.

The results of this work were used as the basis of another study to determine the 'no-effect level' of ethylidene gyromitrin (Niskanen, Pyysalo, Rimaila-Pärnänen & Hartikka, 1976). It would be of value to extend these studies on the toxicity of false morels to the investigation of false-morel preparations containing both ethylidene gyromitrin and other hydrazones.

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TOXICITY OF A MONILIFORMIN-PRODUCING STRAIN OF *FUSARIUM MONILIFORME* VAR. *SUBGLUTINANS* ISOLATED FROM MAIZE

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Abstract—Pure cultures of a very toxic strain of *Fusarium moniliforme* Sheldon var. *subglutinans* Wr. & Reink., isolated from maize (*Zea mays* L.) and cultured on autoclaved maize, were fed to ducklings and rats. Ducklings consuming 0·5–2·0 g of mouldy meal died within 2 hr and those given doses of material derived from a methanol extraction of chloroform-extracted meal died in less than 60 min. The culture material was also acutely toxic to rats, males being more sensitive than females to the toxic effects of low dietary levels of mouldy meal. Semi-quantitative analysis based on UV spectrophotometry of methanol extracts of this mouldy meal revealed very high levels of moniliformin (up to 11·3 g/kg) in the toxic mouldy meal, and bioassay in ducklings demonstrated a dose–effect relationship for batches of culture material containing different levels of the toxin. The oral LD_{50} of chemically pure moniliformin was 3·68 mg/kg in 7-day-old ducklings and 50·0 and 41·57 mg/kg, respectively, in male and female inbred BD IX black rats (c.100 g), with both species showing progressive muscular weakness, respiratory distress, cyanosis, coma and death. Autopsy of the rats showed acute congestive heart failure and the histological lesions, although non-specific, were characterized particularly by acute focal myocardial degeneration and necrosis, while severe cloudy swelling and scattered single-cell necrosis occurred in the liver, kidney, pancreas, adrenal glands, gastric mucosal glands and crypts of the small intestine. The myocardium was also the site of the major lesions in rats fed *F. moniliforme* var. *subglutinans* culture material. Acute hydropic degeneration, focal Zenker's necrosis, myolysis and fibrosis occurred in all the experimental groups to an extent varying with the duration of intake and the dietary level of the mouldy feed. Rats on a diet containing 8% culture material showed the most extensive lesions. Less severe myocardial lesions occurred in rats fed a diet containing 2% culture material.

INTRODUCTION

Fusarium moniliforme Sheldon is the most prevalent fungus on maize (*Zea mays* L.) in South Africa and many other maize-producing countries (Kellerman, Marasas, Pienaar & Naudé, 1972). Under experimental conditions, *F. moniliforme* has been found to be acutely toxic to chickens (Cole, Kirksey, Cutler, Doupnik & Peckman, 1973; Fritz, Mislivec, Pla, Harrison, Weeks & Dantzman, 1973; Marasas & Smalley, 1972; Sharby, Templeton, Beasley & Stephenson, 1973), to donkeys and horses (Kellerman *et al.* 1972; Marasas, Kellerman, Pienaar & Naudé, 1976; Wilson & Maronpot, 1971), to ducklings (Scott, 1965), to mice (Arai & Ito, 1970; Korpinen & Ylimäki, 1972; Ueno, Ishikawa, Nakajima, Sakai, Ishii, Tsunoda, Saito, Enomoto, Ohtsubo & Umeda, 1971; van Rensburg, Purchase & van der Watt, 1971) to pigs (Peters, 1904), to rabbits (Joffe, 1960; Joffe, Palti & Arbel-Sherman, 1973) and to rats (Marasas & Smalley, 1972; Smalley, Marasas, Strong, Bamburg, Nichols & Kosuri, 1970). The causative role of *F. moniliforme* in field outbreaks of equine leukoencephalomalacia has been established beyond doubt (Marasas *et al.* 1976; Wilson, 1971; Wilson & Maronpot, 1971; Wilson, Maronpot & Hildebrandt, 1973).

Considering the widespread occurrence of *F. moniliforme* on a staple human and animal foodstuff such as maize, and the number of strains that have been shown to be toxic, surprisingly little information is available about the chemical nature of the mycotoxins produced by this fungus. A few isolates have been found to produce low levels of the oestrogenic metabolite zearalenone (Hacking, Rosser & Dervish, 1976; Kotsonis, Smalley, Ellison & Gale, 1975; Lásztity & Wöller, 1975; Mirocha & Christensen, 1974; Mirocha, Christensen & Nelson, 1969). A cytotoxic metabolite, fusariocin A, with an ip LD_{50} of 2·88 mg/kg in mice, has been isolated and partially characterized from a Japanese isolate of *F. moniliforme* by Arai & Ito (1970).

A new mycotoxin, moniliformin, was recently found to be elaborated by an isolate of *F. moniliforme* obtained from maize damaged with southern leaf blight in the USA (Cole *et al.* 1973), but the original isolate soon lost the ability to produce the toxin. Moniliformin was eventually purified from another

strain of *F. moniliforme* (ATCC 12763) and was identified by X-ray crystallography as the potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione (Springer, Clardy, Cole, Kirksey, Hill, Carlson & Isidor, 1974). Total synthesis of moniliformin has been achieved by Springer *et al.* (1974) and some spectral data have been reported by Lansden, Clarkson, Neely, Cole & Kirksey (1974). The oral LD_{50} of moniliformin in cockerels is 4 mg/kg and it has marked phytotoxic and growth-regulating effects in plants (Cole *et al.* 1973). Moniliformin does not inhibit the incorporation of ^{14}C-labelled leucine into protein by rabbit reticulocytes (Ueno & Shimada, 1974), but some evidence of interaction with DNA has been presented (Lansden, 1973).

Little is known about the occurrence and distribution of *F. moniliforme* Sheldon var. *subglutinans* Wr. & Reink, because of its frequent mis-identification as *F. moniliforme* (Booth, 1971). The same applies to *F. moniliforme* Sheldon var. *anthophilum* (A. Br.) Wr. which is recognized as a distinct variety by some taxonomists (Gerlach, 1970; Joffe, 1974; Joffe *et al.* 1973; Wollenweber & Reinking, 1935). The only published information on the toxicity of these two varieties is the report by Joffe *et al.* (1973) that extracts of two isolates of each variety caused toxic reactions on rabbit skin. The chemical nature of the metabolite(s) responsible for the dermal toxicity was not determined.

During a study of toxic fungi in South African maize, an extremely toxic culture of *F. moniliforme* var. *subglutinans* was isolated from maize grown in the Transkei. This paper described the morphology, taxonomy, toxicity, pathology and moniliformin-producing ability of the isolate of *F. moniliforme* var. *subglutinans* from Transkeian maize.

EXPERIMENTAL

Isolation of fungi. A sample of mouldy maize cobs was collected in Zazulwana Location, near Butterworth, Transkei, during September 1975. *Fusarium moniliforme* var. *subglutinans* was readily isolated in pure culture by transferring small pieces of a conspicuous fungal growth on insect-damaged kernels to plates of 1·5% malt-extract agar containing 100 mg sodium novobiocin/litre. Single-spore isolates were made from one of the primary colonies obtained in this way. One of these single-spore isolates was designated MRC 115 and mass transfers from this isolate were maintained as stock cultures on potato-dextrose agar slants.

Preparation of bulk cultures of MRC 115. Conidial suspensions from stock cultures were used to inoculate the surface of 50 ml solidified potato-dextrose agar in 250-ml Erlenmeyer flasks. These cultures were incubated at 25°C until profuse sporulation occurred. Conidial suspensions for use as inoculum were prepared by adding 150 ml sterile water containing glass beads to each flask and rotating to dislodge the conidia. Yellow maize kernels in 2-litre glass fruit jars (400 g maize and 400 ml distilled water/jar) were autoclaved at 121°C and 103 kPas for 1 hr on each of two consecutive days. The autoclaved maize was inoculated with conidial suspensions (2 ml/jar) by means of a hypodermic syringe and incubated in a growth room at 25 ± 2°C in the dark for 21 days. The contents of the jars were then dried at 45°C for 24 hr and ground in a laboratory mill and the resulting meal was stored at 5°C until use. Three different batches of culture material (MRC 115/1, 115/2 and 115/3) were prepared in this way.

Chemical analysis of culture material for moniliformin. In the first approach, the mouldy maize meal was extracted with chloroform–methanol (1:1)* for 20 hr in a Soxhlet apparatus. However, chemical analysis for moniliformin and bioassay of the crude extract and the extracted maize revealed that this removed only part of the toxic component. In subsequent experiments, the mouldy meal (100 g) in two thimbles (41 × 123 mm) was extracted in a Soxhlet apparatus with chloroform for 30 hr on a waterbath at 90–95°C to remove lipids and red pigments from the meal. The chloroform was removed from the sample, and the chloroform extracts were combined and concentrated under reduced pressure. Bioassay and chemical analysis indicated the absence of moniliformin from this extract. The residual maize from each thimble was extracted with methanol (500 ml) for 48 hr on a waterbath at 93–95°C. The methanol extracts were combined in a 1-litre volumetric flask and made up to that volume by the addition of methanol. The ultraviolet absorption of this solution was determined after appropriate dilution (1 in 250 for these extracts). The concentration of moniliformin in the mouldy meal was computed from its extinction coefficient values λ_{max}^{MeOH} 259 and 231 nm (ϵ, determined as 4800 and 20,300, respectively), the molecular formula for moniliformin being taken as C_4HO_3Na. For measurement of the absorption of these samples, the base-line was set as zero at 290 nm. This spectrophotometric method is not generally applicable to methanol extracts from all suspected moniliformin-containing fungal cultures, since some of these contain metabolites with very strong interfering absorption in the crucial region of 260 and 230 nm. The method therefore lacks sensitivity for the analysis of moniliformin in naturally contaminated samples.

The methanol was evaporated from the above extract and from the extracted culture material at 40°C under reduced pressure. Bioassay of these residues clearly established that the material derived from the methanol extraction contained all of the toxicity and that this procedure removed practically all of the toxic factors from the mouldy meal. A typical extraction procedure is shown in the Fig. 1.

A portion (600 mg) of the foregoing methanol extract was separated preparatively by chromatography on six Merck silica chromatoplates (thickness 2 mm, 20 × 20 cm). The chromatoplates were developed twice in chloroform–methanol (3:2) and dried in the air, and the moniliformin was detected under ultraviolet illumination at 254 nm. A dark absorbent band around R_F 0·65 was scraped off and the moniliformin was eluted with chloroform–methanol (1:1). The material was crystallized from methanol–water and the amount of moniliformin was estimated spectrophotometrically as outlined above. A solution of isolated moniliformin in water was analysed by atomic absorption (Perkin Elmer Model 303 spectrometer) for Na^+ and K^+ using lines 589·0 nm and

*Throughout this paper solvent ratios are volume/volume.

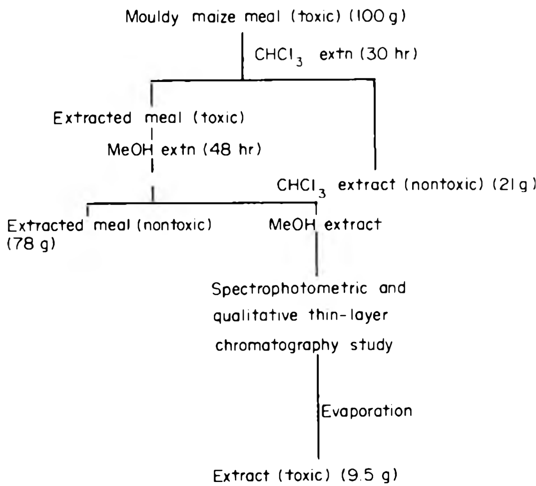

Fig. 1. Scheme for the extraction and fractionation of *F. moniliforme* var. *subglutinans* MRC 115/2.

766·5 nm, respectively. Na^+ and K^+ were present in a ratio of 18:1, indicating that the solution contained a mixture (18:1) of the sodium and potassium salts. Moniliformin applied to a precoated silica layer chromatoplate (thickness 0·25 mm) appears at R_F 0·45 after development in chloroform–methanol (3:2), but the R_F value of moniliformin is concentration-dependent and decreases noticeably at relatively high concentrations (> 10 μg). Therefore, it is essential to use reference standards of pure moniliformin for its analysis in suspected samples. A stock solution of moniliformin (1 mg/ml) used for this purpose remains quite stable when stored in the dark at 0°C. The presence of moniliformin on chromatoplates can be visualized by spraying with a solution of 0.32% 2,4-dinitrophenylhydrazine in 2 N-HCl; the moniliformin appears as a red-brown spot after the chromatoplate has been heated for 10–15 min at 100–110°C. Pure moniliformin can be detected on silica thin-layer chromatoplates containing fluorescent indicator to a minimum level of 0·2 μg by its absorption at 254 nm and by reaction with the above chromogenic agent. These basic methods of detection can be used for the semi-quantitative estimation of moniliformin present in fungal extracts (Rabie, Lübben, Louw, Rathbone, Steyn & Vleggaar, 1978).

Chemically pure moniliformin (the sodium and potassium salt of 1-hydroxycyclobutene-1-ene-3,4-dione) for use in LD_{50} determinations was isolated from the culture material of *F. moniliforme* var. *subglutinans* MRC 115 (Steyn, Thiel & Van Schalkwyk, 1978).

Acute toxicity trials in ducklings

The LD_{50} of chemically pure moniliformin was determined in 7-day-old Pekin ducklings by the method of Weil (1952). Thirty ducklings were weighed before the start of the experiment, divided randomly into five groups and caged in groups of six. They were dosed once only by crop intubation with moniliformin dissolved in sterile water in appropriate concentration to provide a dose of 0·5, 1·375, 3·683 or 10·0 mg/kg body weight in the dosage volume of 5 ml/kg. Ducklings in the control group received the same volume of sterile distilled water. Times of dosing and of death, clinical signs and death rates were recorded.

Diets containing three different batches of *F. moniliforme* var. *subglutinans* culture material were prepared by mixing the mouldy maize meal with an equal weight of commercial chicken mash. Control diets contained 50% maize meal prepared from autoclaved, non-inoculated yellow maize and 50% chicken mash. These diets were fed *ad lib.* to groups of four Pekin ducklings, either 1 or 7 days old. The ducklings and their respective feeds were weighed at the beginning of each experiment and feed intake values were calculated from the amount of feed remaining at the conclusion of each test.

The toxicity of the three batches of culture material was also assayed by treating groups of four day-old ducklings with extracts prepared from 50-g samples of the different batches of mouldy meal. These samples were extracted with chloroform, the extract was discarded and the meal was extracted with methanol. The methanol was completely removed from the extract under reduced pressure, 5 g of the residue was dissolved in 5 ml distilled water and the solution was administered by crop intubation in a volume of 0·25 ml/duckling. Each duckling was dosed only once, the dosage being equivalent to 2·5 g of mouldy meal. Controls were dosed with distilled water. After dosing, the animals were kept under continuous observation and the times of death were recorded accurately.

Toxicity trials in rats

The solutions of chemically pure moniliformin in sterile distilled water were administered also to groups of five individually caged male and female inbred BD IX black rats, weighing about 100 g, for LD_{50} determinations according to the method of Weil (1952). The rats were weighed before the start of the experiment and given a single dose of 25·0, 39·68, 62·99 or 100·0 mg/kg body weight, in each case in a volume of solution equivalent to 5 ml/kg. Rats of the control group received the same volume of sterile distilled water. The times of dosing and of death, clinical signs and death rates were recorded.

Batch MRC 115/1 of the *F. moniliforme* var. *subglutinans* culture material was mixed with commercial rat mash to produce diets containing 2, 4, 8, 16 and 32% (w/w) of mouldy meal. The control diet consisted of rat mash only. These diets were fed *ad lib.* for 12 wk to groups of four male and four female inbred BD IX black rats housed two to a cage under standard conditions. The rats were weighed individually at weekly intervals. After they had become accustomed to the diets for 2 wk, feed consumption was determined by weighing both rats and residual feed daily over a 5-day period. Feed cups were provided with saucers and a perforated metal disc was placed on top of the feed to minimize feed wastage.

Autopsies were performed on all rats that died and on all the survivors which were destroyed by decapitation at the termination of the experiments, 7 days for the LD_{50} determination and 12 wk for the cultured material. Specimens of all the organs were collected in 10% buffered formalin, trimmed, embedded in paraffin wax, sectioned at *c.*6 μm, stained with haematoxylin and eosin and examined under a light microscope.

RESULTS AND DISCUSSION

Morphology and taxonomy of strain MRC 115

Mouldy maize cobs collected in the Transkei had many broken and insect-damaged kernels exhibiting a conspicuous pink discoloration. Microscopic examination of the pink fungal growth revealed it to be a mixture of one-celled, spindle-shaped microconidia, 10–14 × 2·5–4·0 μm, and fusoid, thin-walled, 3–5-septate macroconidia, 25–40 × 2·5–3·5 μm. The pink fungus was readily isolated in pure culture by direct transfer of conidia to plates of 1·5% malt-extract agar containing sodium novobiocin. A single-spore isolate from one of the colonies obtained in this way was designated strain MRC 115.

Cultures of MRC 115 on potato-dextrose agar at 25°C grow rapidly and give rise to dark purple colonies with floccose aerial mycelium. The aerial mycelium soon becomes powdery, due to the development of numerous microconidia in false heads. Microconidia are formed on short phialides from branched conidiophores and are spindle-shaped or ovoid with both ends rounded or slightly flattened at the base, hyaline, one-celled or one-septate, 6·5–16·0 × 2·0–4·0 μm. In cultures incubated at 20°C under near ultraviolet-light radiation for 12 hr/day and in the dark for 12 hr/day, numerous orange-coloured macroconidial sporodochia develop in the aerial mycelium and on the surface of the agar within 14 days. Macroconidia are fusoid, straight or curved, thin-walled, with a pedicellate basal cell and curved apical cell, hyaline, 3–5-septate, 32–48 × 3·5–4·0 μm (typically with three distinct septa and 36 × 4 μm). Chlamydospores are absent.

Isolate MRC 115 belongs in the Section Liseola but is not *F. moniliforme* because the microconidia are not formed in chains from simple phialides (Booth, 1971). The culture was kindly examined by Dr. C. Booth of the Commonwealth Mycological Institute, Kew, England, who suggested that it might represent *F. moniliforme* var. *anthophilum* (A.Br.) Wr. Although Booth (1971) included this as a synonym of *F. moniliforme* var. *subglutinans* Wr. & Reink., some other authors have maintained var. *anthophilum* as a separate variety because of the production of pyriform as well as spindle-shaped microconidia (Gerlach, 1970; Joffe, 1974; Joffe *et al.* 1973; Wollenweber & Reinking, 1935). In order to compare MRC 115 with these two varieties of *F. moniliforme*, the following cultures were obtained through the courtesy of Dr. H. Nirenberg from the Deutsche Sammlung für Mikroorganismen (DSM) at the Institut für Mykologie of the Biologische Bundesanstalt für Land- und Forstwirtschaft (IMB), Berlin, Germany:

DSM-IMB 62267, *F. moniliforme* var. *anthophilum*, *Zea mays*, Iran, Gerlach.

DSM-IMB 62268, *F. moniliforme* var. *subglutinans*, *Zea mays*, Iran, Gerlach.

The isolate of *F. moniliforme* var. *anthophilum* (DSM-IMB 62267) was found to produce distinctly pyriform, one-celled microconidia, 5·5–11·0 × 2·5–5·0 μm, in addition to the spindle-shaped microconidia. The production of these pyriform microconidia is such a distinctive characteristic that var. *anthophilum* could probably be considered as a separate species. These characteristic pyriform microconidia were not found in *F. moniliforme* var. *subglutinans* (DSM-IMB 62268) or in strain MRC 115 and these two isolates were identical in all essential respects. Strain MRC 115 was accordingly identified as *F. moniliforme* Sheldon var. *subglutininans* Wr. & Reink.

Moniliformin determinations

Spectrophotometric analysis of methanol extracts of the three batches of culture material (MRC 115/1, 2 and 3) indicated the presence of 9·7, 11·3 and 6·1 g moniliformin/kg, respectively. In terms of the normally expected yields of mycotoxins, these moniliformin yields are extremely high, particularly in view of the fact that the oral LD_{50} of moniliformin in cockerels is as low as 4·0 mg/kg (Cole *et al.* 1973).

Acute toxicity trials in ducklings

All three batches of culture material caused 100% mortality within 48 hr when fed to groups of day-old ducklings. Since it was observed that day-old ducklings did not start feeding directly, and stopped feeding after the consumption of very small amounts of mouldy material, diets containing culture material were also fed to 7-day-old ducklings. Although the larger ducklings also consumed only small quantities before refusing feed, the amounts of toxic meal ingested at the first feeding were sufficient to kill them very rapidly (in 87–177 min; Table 1). The mean time of death decreased as the calculated amount of moniliformin (based on spectrophotometric determinations in methanol extracts) increased, and a negative linear relationship ($r = -0·71$) was found between moniliformin consumption and log time of death (Fig. 2).

A dose–effect relationship was established by dosing the methanol fractions of chloroform-extracted mouldy meal to ducklings. The individual times of death in the dosing experiment varied from 18 to 67 min (Table 2), and a negative linear relationship ($r = -0·55$) was again found between the calculated amount of moniliformin present in the methanol fractions and log time of death (Fig. 2). The dosage levels of this preparation were unfortunately extremely high (Table 2), since for crystalline moniliformin a dose of 40 mg/kg is sufficient to cause the death of cockerels within 45 min (Cole *et al.* 1973). At the time of the experiment, the amounts of moniliformin present in the fractions were, however, presumptive.

Toxicity trials in rats

The culture material of *F. moniliforme* var. *subglutinans* (batch MRC 115/1) proved to be extremely toxic to rats (Table 3) and negative linear relationships were found between the percentage of mouldy meal in the diet and log time of death in the case of males ($r = -0·87$) as well as females ($r = -0·76$) (Fig. 3). Diets containing 8% of mouldy meal caused marked reductions in liveweight gain (Tables 3 & 4) and feed consumption (Table 4). All the male and three of the four female rats that received the 8% diet died (Table 3). Male rats appeared to be somewhat more susceptible than females and in the groups fed 4% mouldy meal three of the four males, but no females, died. In the three female groups in which deaths occurred (those on the 8, 16 and 32% dietary

Table 1. *Acute toxicity of* F. moniliforme *var.* subglutinans *culture material to ducklings*

Batch no.	Moniliformin content (g/kg maize meal)*	Mean live-weight (g)	Mean feed intake (g/duckling)	Moniliformin intake† (mg/kg)	Mean time of death and range (min)
MRC 115/1	9·7	134·25	1·0	36·1	136·0 (107–177)
/2	11·3	137·25	3·75	154·4	89·75 (87–95)
/3	6·1	146·75	2·5	50·9	112·75 (97–143)

*Diets containing 50% mouldy maize meal and 50% commercial chicken mash were fed to groups of four 7-day-old Pekin ducklings.
†Calculated from the spectrophotometrically determined moniliformin content of the respective batches of culture material.

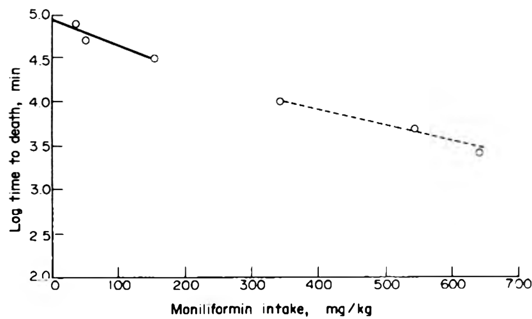

Fig. 2. Linear dose-response relationship between calculated moniliformin intake by ducklings and log time of death following the feeding of mouldy meal containing *F. moniliforme* var. subglutinans culture material (—; $r = -0{\cdot}71$) or the intubation of methanol extracts (---; $r = -0{\cdot}55$).

levels) the mean times of death were, however, shorter than those in the corresponding males (Table 3).

It appears that mouldy meal levels of 8% and higher rendered the diets highly unpalatable, while diets containing 4 or 2% mouldy meal were consumed relatively well (Table 4). On the basis of the chemical determination of the moniliformin content of batch MRC 115/1 (9·7 g/kg) and the mean daily feed consumption (Table 4), the cumulative amounts of moniliformin consumed over a 12-wk period by the rats fed 2% mouldy meal can be calculated as 286·8 mg/rat and 210·2 mg/rat for males and females respectively. In order to have tolerated these remarkably large amounts of moniliformin, these rats must have been able to detoxify the relatively low levels of moniliformin in the feed effectively. A level of 4% mouldy meal in the diet, however, proved to be lethal to male rats, although all the females survived a calculated consumption of 368·3 mg moniliformin/rat.

Determination of LD_{50} values

The single-dose oral LD_{50} values (with 95% confidence limits) for chemically pure moniliformin were 3·68 (2·41–5·75) mg/kg in ducklings, 41·57 (33·14–52·12) mg/kg in female rats and 50·00 (38·5–64·94) mg/kg in male rats. The clinical signs of intoxication were similar in both species and were characterized by a rapidly progressive muscular weakness, sternal recumbency, abdominal respiration, respiratory distress, terminal coma and death. No convulsions occurred at any stage. In the case of the ducklings, all deaths occurred within 1 hr whereas, in the case of the rats, the deaths extended over 3 hr. All animals that did not die within this period recovered completely and were clinically normal within 12 hr of the start of the experiment.

Pathology of treated rats

Mouldy meal toxicity trial. Death rates in the rats treated with the *F. moniliforme* var. *subglutinans* culture material are presented in Table 3. At autopsy no changes were evident in the control group, in the males or females fed 2% mouldy meal or in the survivors and two of the animals that died in the group fed 4% mouldy meal. In the latter group, focal haemorrhage occurred on the rugae in the gastric fundus in one of the males.

Severe generalized atrophy, particularly in the groups fed 8 or 16% mouldy meal, and a generalized venous congestion and cyanosis were evident in all the animals that died. The most conspicuous change

Table 2. *Bioassay of methanol fractions of* F. moniliforme *var.* subglutinans *culture material in ducklings*

Batch no.	Mean live-weight (g)	Moniliformin administered*		Mean time of death and range (min)
		mg/dose	mg/kg	
MRC 115/1	45·5	24·25	544·9	43·25 (29–67)
/2	44·0	28·25	642·0	34·5 (18–50)
/3	44·5	15·25	342·7	56·25 (37–64)
Control	43·5	0·0	0·0	—

*Methanol fractions of chloroform-extracted culture material were dissolved in water and administered by crop intubation to groups of four day-old Pekin ducklings. The amount of moniliformin in the administered dose was equivalent to that present in 2·5 g of culture material, as determined spectrophotometrically in each batch (Table 1). Controls were dosed with water.

Table 3. *Toxicity of* F. moniliforme *var.* subglutinans *culture material to rats*

Dietary concn of mouldy meal* (%)	Mean terminal weight of rats (g)		No. of deaths†		Mean time of death (days‡)	
	Males	Females	Males	Females	Males	Females
0 (control)	237·2	182·5	0	0	—	—
2	230·7	172·2	0	0	—	—
4	196·0	137·7	3	0	61·7	—
8	—	85·0	4	3	49·2	42·3
16	—	—	4	4	18·0	14·5
32	—	—	4	4	11·2	10·5

*Diets containing commercial rat mash and the specified levels of mouldy maize meal (batch MRC 115/1) were fed to groups of BD IX rats for 12 wk. Control diet consisted of rat mash only.
†In groups consisting initially of four males or four females.
‡From the start of feeding.

in all the groups was the occurrence of focal or linear erosions and mild intramucosal haemorrhage on the rugae of the gastric fundus in approximately 50% of the rats. The stomach was either empty or well filled with partially digested food. The myocardium had a distinctly parboiled appearance in two animals fed the 8% mouldy meal. The lungs were usually mildly emphysematous, while focal pulmonary haemorrhage occurred in two male and two female rats fed 32% mouldy meal.

Histologically, the generalized atrophy was particularly severe in the groups fed 8 or 16% mouldy meal, but it was also evident in the group fed 32%. It was accompanied by a generalized venous congestion in the animals that died.

In all the groups, the most conspicuous and probably the most important lesions occurred in the myocardium. Depending on the time to death, myocardial degeneration, necrosis and fibrosis were evident. Acute degenerative lesions characterized by a pronounced granularity of the sarcoplasm and, in three of eight rats, by focal Zenker's degeneration and necrosis, particularly in the papillary muscles and left ventricular subendocardium, occurred in the rats fed 32% mouldy meal. In those fed the 16% level, the lesions were very similar, although Zenker's necrosis was more prominent. The most pronounced lesions occurred in the rats fed 8% mouldy meal. Widely distributed focal Zenker's necrosis commonly manifesting fragmentation and macrophage infiltration alternating with focal areas showing myolysis and fibrosis occurred in the papillary muscles and left ventricular subendocardium but also in the right ventricular wall and interventricular septum. In the rats fed the 4% level, similar changes occurred in the males that died, while the one male and four female survivors manifested similar but much less extensive lesions. Focal areas of Zenker's necrosis, occasional macrophage aggregates and a few focal areas of early myocardial fibrosis occurred in 75% of males and females. No changes were evident in the remaining rats.

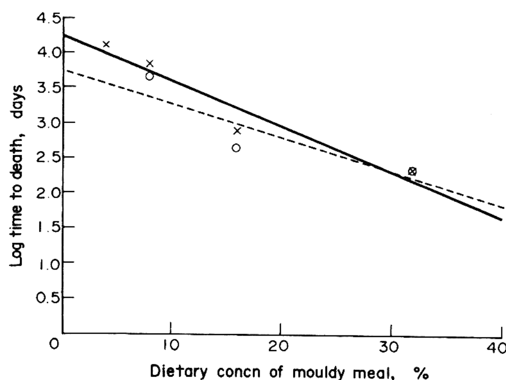

Fig. 3. Linear dose-response relationship between concentration of mouldy meal in the diet and log time of death in male (— × ; $r = -0{\cdot}87$) and female (---○; $r = -0{\cdot}76$) rats treated with *F. moniliforme* var. *subglutinans*.

The lesions in the other organs in the rats fed 2–32% mouldy meal were commonly non-specific and fairly mild, the only prominent features being a moderate to severe nephrosis and focal tubular necrosis in the renal cortex, focal congestion and haemorrhage accompanied by superficial mucosal necrosis in the glandular stomach, moderately severe emphysema and occasional intra-alveolar haemorrhage in the lungs, mild disseminated single-cell necrosis in the pancreas, fatty changes and occasional scattered single-cell necrosis in the adrenal cortex and testicular degeneration. An acute mycotic necrotic gastritis and duodenitis occurred in one male fed 32% mouldy meal. Apart from the myocardial lesions, no specific changes occurred in the organs of the rats fed 2% mouldy meal. No comparable lesions were observed in any of the control rats.

Single-dose oral toxicity (LD_{50}) *study.* Rats given a single dose of the chemically pure toxin showed, at autopsy, generalized congestion and cyanosis, a mildly swollen liver and occasionally a straw-coloured pericardial and thoracic effusion. No characteristic or diagnostic macroscopic lesions occurred. Although of similar nature, the histological lesions in rats varied in severity according to the dosage rate in the LD_{50} determination. Apart from mild myocardial lesions, recovered animals were virtually normal. The detailed histological findings were as follows:

(i) *Control group.* Mild pneumonitis and a mild subacute lymphocytic bronchitis and peribronchitis were the only apparent histological lesions.
(ii) *Experimental group,* 100·0 *mg/kg.* Generalized congestion accompanied by severe, acute

Table 4. *Moniliformin intake of BD IX inbred rats fed diets containing different levels of* F. moniliforme *var.* subglutinans *culture material*

Dietary concn of mouldy meal (MRC 115/1) (%)	Sex	Mean live-weight* (g)	Mean feed intake† (g/rat/day)	Mean moniliformin intake (mg/kg/day)‡	No. of deaths§
0	M	202·75	19·1	0	0
	F	153·25	14·7	0	0
2	M	205·0	17·6	16·6	0
	F	151·5	12·9	16·5	0
4	M	204·0	18·2	34·6	3
	F	133·25	11·3	32·9	0
8	M	162·5	6·9	32·5	4
	F	119·25	5·3	34·5	3

*On day 15 of feeding.
†Mean daily intake over a 5-day period from day 15 to 20.
‡Calculated on the spectrophotometric determination of the moniliformin content of the mouldy meal as 9·7 g/kg (Table 1).
§In groups consisting initially of four males or four females.

degenerative changes and limited necrosis occurred, particularly in the liver, kidney, myocardium, adrenal gland and pancreas and occasionally in the gastric mucosal glands and small intestinal crypts. The liver manifested a severe hydropic degeneration and cloudy swelling of the hepatocytes resulting in sinusoidal obliteration throughout the liver. Scattered single necrotic cells without a particular lobular distribution and characterized by a pyknotic nucleus and a dense, contracted, deeply eosinophilic cytoplasm occurred throughout the liver. A severe nephrosis characterized by extensive cytoplasmic vacuolation and a markedly increased prominence of the basally arranged mitochondria occurred throughout the renal cortex and medulla. Focal hyaline droplet degeneration and single necrotic cells similar in appearance to those in the liver, occurred in the proximal convoluted tubules. In the myocardium, the muscle fibres throughout showed a reduced staining capacity and a distinct fine granularity most probably attributable to swollen mitochondria. Focal Zenker's necrosis affecting single fibres or small, well-demarcated groups of fibres occurred in two of the five cases, particularly in the papillary muscles but also in the wall of the left ventricle and interventricular septum. Extensive cytoplasmic vacuolation characteristically seen as distinct, single, large vacuoles, occurred in the adrenal zona reticularis. Scattered single necrotic cells similar in appearance to those in the liver, occurred throughout the adrenal cortex, focally in the pancreas and in the lower third of the gastric glands and small intestinal crypts. Pulmonary alterations noted were confined to a mild alveolar emphysema and a moderate to severe congestion.

(iii) *Experimental group*, 62·9 *mg/kg*. In this group the lesions were similar but more severe than in (ii). Scattered single cell necrosis in the various organs was more common, while focal Zenker's necrosis and focal myocardial haemorrhage occurred in 60% of the cases. The longest-surviving rat among the animals that died in all the groups showed randomly distributed, focally disseminated areas of necrosis and periacinar fatty changes in the liver, more extensive focal tubular necrosis in the kidneys, restricted lymphoid necrosis in the splenic germinal centres and focal single-cell necrosis characterized by karyorrhexis in the lower third of the intestinal crypts.

(iv) *Experimental group*, 39·68 *mg/kg*. In this group there was a distinct difference between the rats that died and the survivors. Although hepatic necrosis was more extensive and focal haemorrhage occurred at the cortico-medullary junction of the kidney in one animal, the changes in the animals that died, resembled the changes in the previous groups. In the animals that survived, there were no changes in the liver, except for a few cells showing severe cytoplasmic vacuolation. The myocardium was either unaffected, showed a few focal aggregates of macrophages surrounding segments of necrotic fibres, or showed focal disseminated areas of Zenker's necrosis, particularly in the papillary muscle of the left ventricle. The necrotic fibres were vacuolated and surrounded by a few macrophages. Single necrotic cells occurred in the proximal convoluted tubules, while one case showed focal tubular dilatation and atrophy of the lining cells of the proximal convoluted tubules in these areas. In this case, round densely basophilic and laminated bodies resembling corpora amylacia occurred in the tubules in the cortico-medullary junction. No significant changes occurred in the other organs.

(v) *Experimental group*, 25 *mg/kg*. In 40% of the rats in this group there were myocardial lesions resembling those in the animals that recovered from treatment with 39·68 mg/kg. Focal renal tubular necrosis was present in only one rat, while numerous basophilic cytoplasmic inclusions in the acinar cells and occasional necrotic acini occurred in the pancreas in one other. No other lesions were seen.

Conclusions

An extremely toxic isolate of *Fusarium moniliforme* var. *subglutinans* has been obtained from maize grown

in the Transkei. This isolate produces massive amounts of moniliformin, the sodium and potassium salt (18:1) of 1-hydroxycyclobut-1-ene-3,4-dione, and proved to be acutely toxic to ducklings and rats. The extremely high yields (c. 10 g/kg) are certainly remarkable for a mycotoxin and so are the short survival times of ducklings and rats following the consumption of minute quantities of culture material. Semi-quantitative analytical methods for the estimation of moniliformin were developed and the results obtained by these methods were supported by the bioassay data.

This is the first report of the production of moniliformin by *F. moniliforme* var. *subglutinans*, the only previously known producers being two strains of *F. moniliforme* (Cole *et al.* 1975; Springer *et al.* 1974). Due to confusion with *F. moniliforme* and possibly with *F. oxysporum* Schlect., knowledge regarding the distribution and prevalence of *F. moniliforme* var. *subglutinans* on maize in Southern Africa is very limited (Doidge, 1938). The only information on the toxicity of *F. moniliforme* var. *subglutinans* that could be found in the literature is the report by Joffe *et al.* (1973) of the dermal toxicity of two isolates to rabbit skin. No indication was given of the chemical nature of the toxin(s) responsible for the skin reaction.

The LD_{50} of moniliformin in ducklings (3·68 mg/kg) closely corresponded to that established by Cole *et al.* (1973) in cockerels (4·0 mg/kg). The tenfold increase in the LD_{50} in rats, however, suggests a marked interspecies variation in susceptibility. There was, furthermore, a difference between the susceptibility shown by male and female rats treated with chemically pure moniliformin and those fed culture material. It is conceivable, however, that the cause of death is different in the acute and subacute intoxication. Acute effects are most likely to be due to a direct generalized cytotoxic effect, while death in the subacute intoxication is probably attributable to myocardial insufficiency resulting from the cumulative effect of continuous ingestion of sublethal amounts of moniliformin. Differences in the death rates in the acute and subacute toxicity trials may be ascribed to inter-sex differences in the competence of specific detoxification mechanisms.

Pathologically there was a close similarity in the nature and distribution of the histological lesions between the animals fed *F. moniliforme* var. *subglutinans* culture material and those dosed chemically pure moniliformin. Clinical signs and the acute degenerative lesions in the myocardium and other organs indicated involvement and possible suppression of ATP-dependent trans-membrane transport mechanisms, resulting in disturbances of intracellular osmolar regulation and the consequent severe intracellular oedema (Smuckler, 1975; Trump, Berezesky, Collan, Kahng & Merger, 1976).

The rapidity of the development of necrosis in the myocardium and the very low levels at which it developed was remarkable. Of more importance, however, is the cumulative effect of repeatedly administered moniliformin and its possible role as an aetiological factor in some of the ideopathic human cardiomyopathies.

This moniliformin-producing strain of *F. moniliforme* var. *subglutinans* was, furthermore, isolated from an area in the Transkei with a high incidence of oesophageal cancer and where food, including maize, has been shown to contain toxic factors (van Rensburg, Purchase, Rose & Roach, 1974) and factors that enhance the incidence of cancer in rats (Purchase, Tustin & van Rensburg, 1975). Considering the evidence of some interaction with DNA (Lansden, 1973), the possible role of moniliformin as an aetiological factor in carcinogenesis should be considered. In the short-term toxicity trials reported in this paper, however, no oesophageal lesions were noted.

Since the importance of moniliformin to man is dependent on a number of unknown factors, it is at present not possible to link this toxin to any ideopathic disease syndrome. The natural occurrence of moniliformin in maize and the distribution and incidence of moniliformin-producing strains of *F. moniliforme* and *F. moniliforme* var. *subglutinans* on maize in South Africa and the Transkei, the acute and chronic toxicity of moniliformin to primates and the mechanisms of action are currently under investigation.

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LEACHING OF RADIONUCLIDES FROM NEUTRON-ACTIVATED TALC IN SERUM AND IN DILUTE HYDROCHLORIC ACID

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Abstract—When neutron-activated talc was suspended in bovine serum, 0·5% of the ^{60}Co and 3·1% of the ^{46}Sc occurring in the sample was found in the supernatant of the centrifuged and filtered suspension. A maximum of about 0·5% of each radionuclide can be attributed to small particles passing through the filter. This indicates that very little ^{60}Co and about 2·5% ^{46}Sc leached from the talc. The findings indicate that from the pulmonary talc burden of about 45 μg observed in a previously conducted pulmonary deposition, translocation and clearance study, the equivalent of about 0·2 or 1·1 μg talc may have leached, depending on whether ^{60}Co or ^{46}Sc data were used for this computation. Leaching effects on the lung burden thus appear to be negligible. When neutron-activated talc was suspended in 2 N-HCl, from 2 to 5% of the induced ^{60}Co, ^{51}Cr, ^{59}Fe and ^{46}Sc was removed, primarily by leaching. This suggests that leaching of radionuclides from talc may take place in the acid environment of the stomach. This leaching and the subsequent absorption of the radionuclide in the intestinal tract could account for the quantities of ^{60}Co found in the urine of the hamsters in the earlier study.

INTRODUCTION

In a pulmonary deposition, translocation and clearance study previously conducted in these laboratories, neutron-activated talc was used to determine talc burdens in hamster tissues and excreta following a single 2-hr inhalation exposure (Wehner, Wilkerson, Cannon, Buschbom & Tanner, 1977). Reported talc burdens were based on direct instrumental measurements of ^{60}Co tracers and, in a number of cases, also of ^{46}Sc tracers, induced in talc prior to the inhalation exposure. A fundamental presumption relevant to the validity of this technique is a direct quantitative correlation between measured radionuclides and talc particles. However, following contact with biological fluids, the tracers may leach from the talc and thus compromise this correlation.

Direct instrumental measurements made in the pulmonary deposition, translocation and clearance study indicated that from 20 to 80 μg talc was retained in hamster lungs and, following mucociliary clearance and subsequent swallowing, from 220 to 940 μg passed through the gastro-intestinal tract and was eliminated in the faeces. If significant leaching from talc occurs in the lung and gastro-intestinal tract, soluble ^{60}Co and/or ^{46}Sc may be absorbed and translocated to other organs or to the urine. If it does represent translocated talc, a calculated talc burden should be similar whether it is based on measurements of ^{60}Co or ^{46}Sc. Since this definitive verification was not always possible due to ^{46}Sc decay, and since without verification measured radionuclide activities due to leaching may be incorrectly interpreted as translocated talc particles, the extent and significance of tracer leaching into body fluids needed to be determined.

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The objectives of the experiment were to estimate leaching of selected radionuclides (^{60}Co, ^{51}Cr, ^{59}Fe and ^{46}Sc) from neutron-activated talc and to evaluate the effect of such leaching on the estimated talc burdens reported for the pulmonary deposition, translocation and clearance study.

EXPERIMENTAL

A 3-g sample of Johnson's Baby Powder®, subsequently called 'talc', was irradiated to an integrated neutron exposure of $1·4 \times 10^{17}$ n/cm^2. Following a decay period of 3 wk, the irradiated talc was thoroughly mixed to provide a homogeneous specific γ-ray activity. Several 0·1-g aliquots were then weighed into 1·4 ml polyethylene vials. Three randomly selected talc-containing vials were subjected to γ-ray analysis on a solid-state Ge(Li) detector to determine the specific counting activities of the radionuclides ^{60}Co, ^{51}Cr, ^{59}Fe and ^{46}Sc.

Solutions of bovine serum (Grand Island Biological Company, Santa Clara, Cal.) and of 2 N-HCl were prepared, and 100-ml aliquots of each were poured into separate 250-ml polyethylene bottles. The pH of the serum was 8 and that of the dilute HCl was 1·8. The serum was treated with sodium azide (0·1 g/litre) immediately after thawing to inhibit bacterial growth during the experiment. Aliquots containing 0·1 g of neutron-activated talc were suspended in the prepared solutions. The suspensions were then placed in a sample-agitating water-bath maintained at 37°C. It was assumed that the solution-to-talc ratio of 100 ml:0·1 g was adequate to make the experiment independent of solution equilibrium effects, and would thus allow maximal leaching of the radionuclides of interest.

The extent of radionuclide leaching into bovine

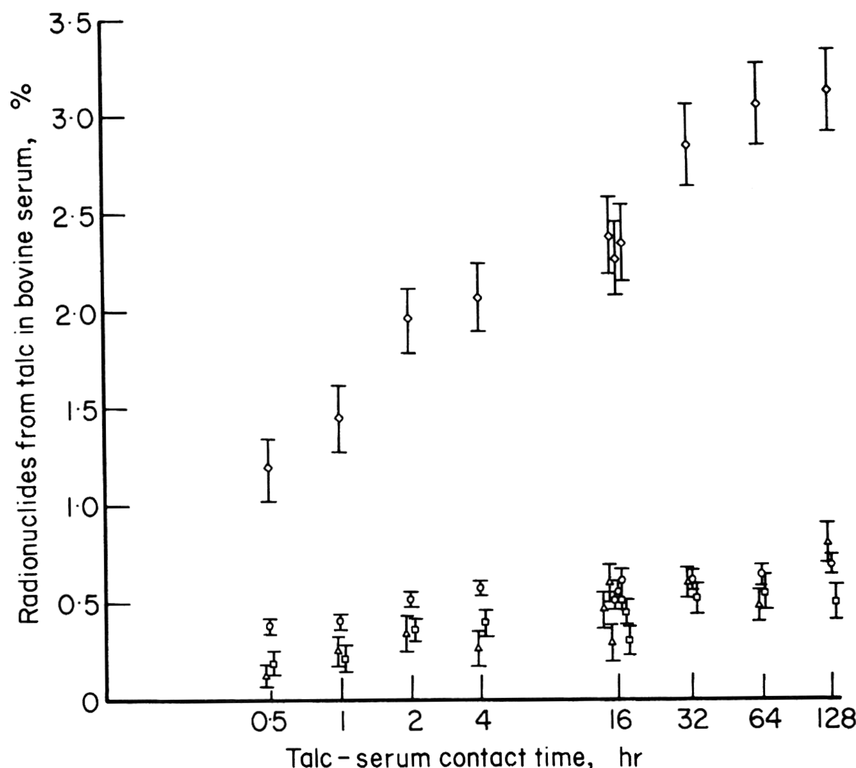

Fig. 1. Removal of ^{46}Sc (◇), ^{60}Co (○), ^{59}Fe (□) and ^{51}Cr (△) from talc suspended in bovine serum (pH 8) in a concentration of 0·1 g/100 ml. Error bars represent the combined error of the standards (mean ± 1s) and supernatant counting errors (1s).

serum or 2 N-HCl as a function of time was investigated by controlling the period of contact between each solution and the talc. Periods for contacting a total of ten aliquots of neutron-activated talc with bovine serum were 0·5, 1, 2, 4, 16, 32, 64 and 128 hr, aliquots for the 16-hr contact time being measured in triplicate to determine experimental precision. Contact periods for a total of three aliquots of neutron-activated talc with dilute HCl were 1, 4 and 128 hr.

At the end of each contact period, the appropriate suspension was removed from the water-bath and centrifuged. A 50-ml aliquot of the supernatant was removed by pipette and filtered through a glass-fibre filter (Gelman Type E) with a rated filtration efficiency of >99% for particle sizes ⩾0·3 μm. The filtered 50 ml of supernatant was analysed on the Ge(Li) detector used previously. After application of appropriate radioactive-decay and counting-geometry corrections, the fraction of each radionuclide that had leached from the talc was determined.

RESULTS AND DISCUSSION

The corrected specific counting activities of the radionuclides ^{60}Co, ^{51}Cr, ^{59}Fe and ^{46}Sc in talc, and in the filtered supernatant solutions of bovine serum and 2 N-HCl are shown in Table 1. Measured specific counting activities for the three randomly selected aliquots of irradiated talc showed close agreement and served as standards.

Computed fractions of each radionuclide removed from neutron-activated talc as a function of the period of contact with bovine serum or 2 N-HCl are shown in Figs 1 and 2, respectively.

Results of contacting neutron-activated talc with bovine serum (Fig. 1) indicate that ^{60}Co, ^{51}Cr, ^{59}Fe and ^{46}Sc were essentially retained by the irradiated talc particles. Retained fractions were >96% for ^{46}Sc and >99% for ^{60}Co, ^{51}Cr and ^{59}Fe, as measured over the entire contact period. Removed fractions of ^{60}Co, ^{51}Cr and ^{59}Fe were within a narrow range around 0·5%. Considering experimental precision and accuracy, the close agreement between these three radionuclide fractions suggests that the removed fraction represents small talc particles which may have escaped filtration. This assumption is in good agreement with the rated >99% efficiency of the glass-fibre filter for particle sizes ⩾0·3 μm. In view of these observations, it is reasonable to conclude that measured fractions of ^{60}Co, ^{51}Cr and ^{59}Fe in filtered bovine serum represented talc particles. Leached fractions of these three radionuclides can be considered negligible.

Preferential removal of ^{46}Sc was observed for all talc-serum exposures. The gradual increase in removed ^{46}Sc as a function of time suggests that leaching effects contributed to the measured ^{46}Sc activities. The observed maximal fraction of ^{46}Sc removed was 3·1% after the 128-hr contact period. Assuming that this value represents maximally extractable ^{46}Sc, and assuming a 0·5% contribution from small talc particles, an estimated fraction of 2·6% represents leached ^{46}Sc.

Alternative explanations for the raised ^{46}Sc levels in the serum supernatant involve the fast neutron

Table 1. *Specific activities for neutron-activated talc and filtered supernatants of bovine serum and dilute HCl*

		Radionuclide activity (cpm/g talc)*						
		^{60}Co		^{51}Cr	^{59}Fe		^{46}Sc	
Sample	Contact time (hr)	1·17 MeVγ	1·33 MeVγ	0·32 MeVγ	1·10 MeVγ	1·29 MeVγ	0·89 MeVγ	1·12 MeVγ
Talc (0·1 g) in vial no. 1	—	2173 ± 12†	1929 ± 11	1597 ± 10	1024 ± 8	671 ± 6	588 ± 6	484 ± 5
2	—	2253 ± 12	1990 ± 11	1677 ± 10	1066 ± 8	695 ± 7	628 ± 6	496 ± 6
3	—	2211 ± 12	1955 ± 11	1607 ± 10	1017 ± 8	681 ± 6	602 ± 6	484 ± 5
Talc (mean ± 1s)	—	2212 ± 40	1958 ± 31	1627 ± 44	1036 ± 27	682 ± 12	606 ± 20	488 ± 7
Bovine serum supernatant	0·5	7·8 ± 0·8	7·8 ± 0·8	2·2 ± 1·0	2·3 ± 0·6	1·3 ± 0·4	5·2 ± 0·6	7·4 ± 0·8
	1	9·4 ± 0·8	7·1 ± 0·8	4·2 ± 1·3	3·1 ± 0·7	0·9 ± 0·4	7·7 ± 0·8	7·9 ± 0·8
	2	10·5 ± 0·8	10·7 ± 0·8	5·7 ± 1·4	3·8 ± 0·7	2·4 ± 0·5	10·1 ± 0·9	11·0 ± 0·9
	4	12·2 ± 1·0	11·2 ± 0·8	4·4 ± 1·4	4·1 ± 0·7	2·7 ± 0·5	12·1 ± 1·0	10·4 ± 0·8
	16‡ (1)	12·5 ± 1·0	10·9 ± 0·8	9·8 ± 1·5	5·2 ± 0·7	2·7 ± 0·5	14·6 ± 1·1	11·4 ± 0·9
	(2)	14·5 ± 1·0	10·8 ± 0·8	7·7 ± 1·5	4·2 ± 0·7	1·2 ± 0·5	13·7 ± 1·1	11·9 ± 0·9
	(3)	10·8 ± 0·8	10·4 ± 0·8	4·6 ± 1·4	4·6 ± 0·7	3·9 ± 0·7	12·5 ± 1·1	12·1 ± 0·9
	32	14·9 ± 1·0	10·5 ± 0·8	9·8 ± 1·6	4·7 ± 0·6	3·9 ± 0·6	16·5 ± 1·0	14·4 ± 1·0
	64	13·7 ± 1·0	13·0 ± 1·0	7·8 ± 1·2	6·4 ± 0·9	3·4 ± 0·6	16·8 ± 1·0	16·2 ± 1·0
	128	16·4 ± 1·0	12·6 ± 1·0	13·1 ± 1·6	4·9 ± 0·7	3·6 ± 0·7	17·8 ± 1·1	16·1 ± 1·1
Dilute HCl supernatant	1	20·6 ± 1·3	20·6 ± 1·0	6·5 ± 2·0	16·4 ± 1·5	12·5 ± 1·2	25·8 ± 1·5	22·3 ± 1·5
	4	24·7 ± 1·3	20·1 ± 1·1	17·1 ± 2·2	25·9 ± 1·6	16·2 ± 1·4	29·6 ± 1·6	27·6 ± 1·4
	128	44·9 ± 1·8	39·8 ± 1·6	33·9 ± 3·0	45·1 ± 2·1	25·7 ± 1·5	30·3 ± 1·7	25·6 ± 1·5

*All values are corrected for radioactive decay and counting geometry. Supernatant solution activities have been converted to cpm/g talc by multiplying with appropriate solution-to-talc ratios.

†Counting error (1s).

‡Determined in triplicate.

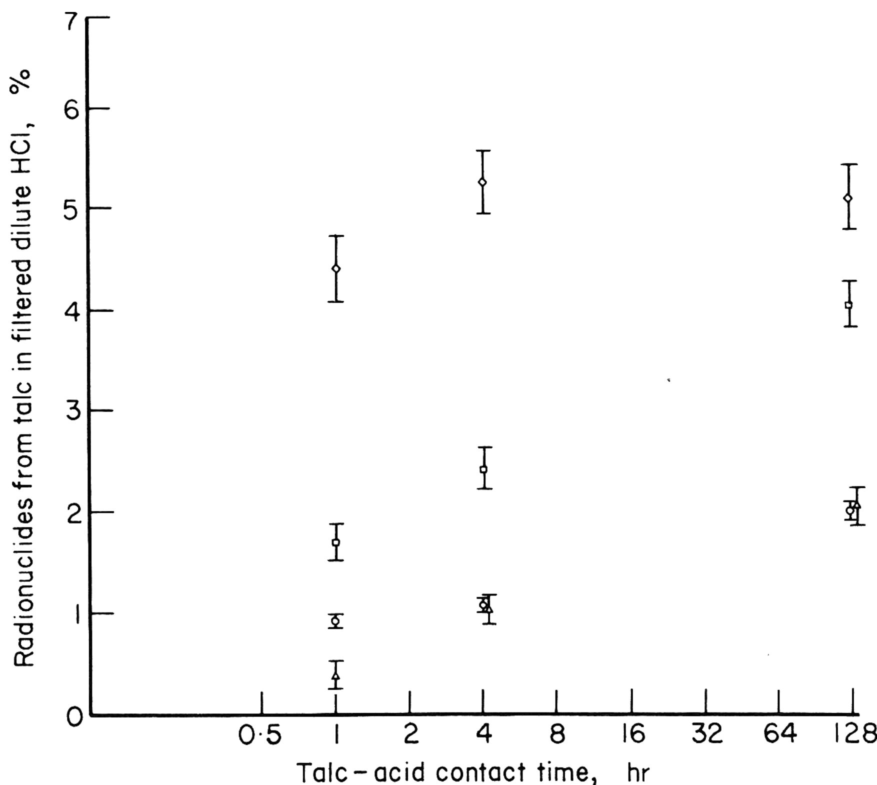

Fig. 2. Removal of ^{46}Sc (◇), ^{60}Co (○), ^{59}Fe (□) and ^{51}Cr (△) from talc suspended in dilute HCl (pH 1·8) in a concentration of 0·1 g/100 ml. Errors bars represent the combined error of the standards (mean ± 1s) and supernatant counting errors (1s).

reaction $^{46}_{22}Ti(n,p)^{46}_{21}Sc$. If significant, this reaction could result in recoiled atoms of ^{46}Sc which might become highly soluble following contact of the irradiated talc with the solution. However, this possibility appears unlikely due to the unfavourable production parameters of ^{46}Ti atomic abundance (8%) and the Ti concentration in this talc (< 200 ppm), as measured by neutron-activation analysis in our laboratory. Also, if recoiled ^{46}Sc atoms were responsible for the increased ^{46}Sc levels in the filtered serum supernatants, the expected fractional removal curve in Fig. 1 would rise sharply within a short exposure time and then level off asymptotically. The data represented by the gradually increasing curve illustrated in Fig. 1 do not support this hypothesis. A second possibility is that the talc contains a high Sc-bearing mineral of small particle size which could preferentially escape the filter or leach.

Assuming a maximal leaching of 0·5% for ^{60}Co and 2·5% for ^{46}Sc, the maximal fraction of these tracers which may have leached from the talc in the hamster lung can be estimated. Using the mean talc burden for hamster lungs (45 μg) demonstrated in the pulmonary deposition, translocation and clearance study (Wehner *et al.* 1977), the fraction of potentially leached ^{60}Co and ^{46}Sc would be equivalent to about 0·2 and 1·1 μg talc, respectively. The estimated quantities of elemental Co and Sc leached from the above talc burden may be calculated from the elemental concentration of Co and Sc in the talc. As measured in our laboratory by instrumental neutron-activation analysis, these values were 64 and 1 ppm for Co and Sc, respectively. The estimated quantities of elemental Co and Sc leached were thus $14·4 \times 10^{-12}$ g Co (from 45×10^{-6} g talc, 64×10^{-6} g Co/g talc and 0·5%) and $1·1 \times 10^{-12}$ g Sc (from 45×10^{-6} g talc, 1×10^{-6} g Sc/g talc and 2·5%).

Table 2 shows the average values for background γ-ray counts with twice the standard deviation for ^{60}Co and ^{46}Sc in various tissues and excreta from control animals, expressed as μg equivalents of talc. It can readily be observed that the estimated quantities of leached ^{60}Co and ^{46}Sc (equivalent to 0·2 and 1·1 μg talc, respectively) are negligible. Thus, quantities of ^{60}Co and ^{46}Sc that may have leached and translocated from the hamster lung would also be negligible and would not affect the results of the pulmonary deposition, translocation and clearance study.

Results of the serum-leaching study suggest that a maximum of 0·5% of the induced radionuclides may have passed into the supernatant as unfiltered talc particles. Contacting neutron-activated talc with 2 N-HCl resulted in greater removals, namely 2–5%. This indicates that all four radionuclides were removed from talc primarily by leaching (Fig. 2). The maximal fraction of ^{46}Sc removed was 5·3%; this was recorded after the 4-hr contact time and appears to be the upper limit of extractable ^{46}Sc. Removed fractions of ^{59}Fe measured in filtered 2 N-HCl supernatants increased linearly as a function of contact time. The maximal fraction removed was 4·1% after 128 hr. Removal of the radionuclides ^{60}Co and ^{51}Cr also showed a linear increase with contact time, the maximal fraction removed from the talc being 2·1% after

Table 2. *Mean γ-ray counts for tissues and excreta of control hamsters, based on ^{60}Co and ^{46}Sc data and expressed in talc equivalents*

Sample	Talc equivalent* (μg) ^{60}Co	^{46}Sc
Lung	2·1 ± 0·9	7·0 ± 5·8
Liver	2·5 ± 1·4	4·6 ± 2·1
Kidneys	2·1 ± 1·7	7·1 ± 2·2
Ovaries	2·2 ± 1·0	5·0 ± 3·8
Carcass	3·0 ± 1·8	8·8 ± 6·8
Urine	1·5 ± 0·4	4·4 ± 2·0
Faeces	4·8 ± 3·4	5·8 ± 2·6

*Values are expressed as means ± 2 SD and are based on ^{60}Co and ^{46}Sc data for the control animals in a pulmonary deposition, translocation and clearance study on talc (Wehner *et al.* 1977).

Table 3. *Estimated quantities of Co and Sc translocated to blood of exposed animals following absorption from gastro-intestinal tract*

Parameter	Co	Sc
Concn of element in talc (ppm)	64	1
Mean quantity (Q) of talc passing through gut* (μg)	455	455
Estimated fraction (f_1) leached in upper gut†	0·01	0·045
Estimated fraction (f_2) absorbed in gut‡	0·3	0·0001
Estimated quantity (T) of element transferred to blood ($\times 10^{-12}$ g)	90	0·002
Equivalent quantity of talc (μg)	1·4	0·002

*Determined by Wehner *et al.* 1977.
†Determined experimentally using ^{60}Co and ^{46}Sc tracers.
‡From report of ICRP Committee II (1960).

128 hr in both cases. The complex behaviour of the ^{46}Sc removal curve, which reaches a maximum after 4 hr, supports a mechanism of removal independent of that of Fe, Co and Cr. The independent behaviour of Sc could be due to the reduction of particles containing high concentrations of Sc to smaller particle sizes or to the leaching of particles containing readily depletable quantities of Sc.

To estimate the leaching effects of the acid environment of the stomach on ^{60}Co and ^{46}Sc, the 1-hr contact data were used. During this contact period, 1% ^{60}Co and 4·5% ^{46}Sc were removed from the talc (Fig. 2). On the basis of the data for gastro-intestinal absorption reported in 1959 by Committee II of the International Commission on Radiological Protection (ICRP), the total quantity of Co and Sc that may have leached from talc and translocated to blood by gastro-intestinal absorption can be estimated using the general equation: $T = Q.f_1.f_2.C$ where T is the estimated quantity of stable element translocated, Q is the estimated quantity of talc passing through the gastro-intestinal tract, f_1 is the estimated fraction of the element (or radionuclide tracer) leached during passage through the gastro-intestinal tract, f_2 is the estimated fraction of the element (or radio nuclide tracer) absorbed from the gastro-intestinal tract and C is the concentration of the element in this sample of talc.

Using the average talc burden for hamster carcasses (455 μg) determined in the pulmonary deposition, translocation and clearance study (Wehner *et al.* 1977), the corresponding equivalent fraction of talc potentially translocated, calculated on the basis of leached ^{46}Sc, is only 0·002 μg (Table 3). This value is far below the levels demonstrated for the control animals in that study (Table 2) and would thus be undetectable. Leaching, therefore, would have had no effect on the ^{46}Sc-determined talc burdens in the pulmonary deposition, translocation and clearance study.

The data on ^{60}Co in Table 3 show that about 90 pg Co may translocate. The corresponding talc equivalent, based on the measurement of ^{60}Co, is about 1·4 μg. Assuming that leached Co translocating to blood would mainly be excreted in the urine, one would expect that the ^{60}Co leached from talc could be measured in the hamster urine. This contention is supported by a comparison of ^{60}Co determinations in the urines of control and exposed animals. The average value for control urines (Table 2) is equivalent to 1·5 ± 0·4 μg talc. The estimated translocation of leached ^{60}Co to blood following gastro-intestinal absorption (Table 3) resulted in a talc burden equivalent of 1·4 μg. Adding the average urine value for the controls (1·5 μg) to that of the experimentally determined equivalent quantity of talc represented by leached ^{60}Co (1·4 μg) yields approximately the values (about 3·5 μg) found in the urine of the exposed animals (Wehner *et al.* 1977). This suggests that leached ^{60}Co accounts to a large extent for the γ-ray counts recorded for the urine of exposed animals.

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THE ABSORPTION OF *p*-TOLUENEDIAMINE BY THE SKIN OF RATS AND DOGS

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Abstract—The cutaneous absorption of *p*-[*Me*-^{14}C]toluenediamine, applied in formulations similar to those normally used for hair dyeing, was investigated in rats and dogs. When rat skin was exposed for 30 min to two different formulations, about 0·2% of the administered amount of *p*-toluenediamine was absorbed in each case. Excretion of the absorbed substance took place predominantly in the urine. At the end of the 24-hr experiment, a residue of 5·2–9·3% of the dose was found in the treated area of rat skin. Experiments with oral and subcutaneous application to the rat also demonstrated rapid elimination of the toluenediamine. Dog skin was exposed to one formulation containing *p*-toluenediamine for 3 hr. It is estimated that about 0·13% of the administered *p*-toluenediamine was absorbed.

INTRODUCTION

A detailed study of the toxicity and carcinogenicity of *p*-toluenediamine in rats was performed by Kinkel & Holzmann (1973), who determined the effects of the substance after it had been applied as for normal use in hair-dyeing. They did not find any toxic effects, but they did not investigate the extent of cutaneous absorption. However, an absorption study in dogs (Kiese, Rachor & Rauscher, 1968) indicated low absorption through the skin. Similar results were found in excretion studies in man (Kiese & Rauscher, 1968). We decided to investigate the cutaneous absorption of *p*-toluenediamine, in rats and dogs. In order to create realistic conditions, the substance was applied in different formulations similar to those normally used in hair-dyeing. Radioactively labelled compounds were used in the present investigation to achieve greater accuracy in determining the extent of cutaneous absorption.

EXPERIMENTAL

Materials

p-[*Me*-^{14}C]toluenediamine was synthesized at the Institute of Chemistry of the Research Center Seibersdorf. The main precursors in the synthesis were [^{14}C]toluene, nitrotoluene and nitroaminotoluene. Radiochemical purity was checked by radiochromatography. Chemical identity was compared with the industrial product by thin-layer chromatography, melting point determination, colorimetry and infra-red spectroscopy. Details of the synthesis are being published elsewhere (Drahowzal & Wiesinger, 1977). Fresh compound was synthesized for each experiment to avoid possible degradation during storage. The solutions were prepared immediately before application.

The other chemicals used were analytical grade, except for the formulation base used for the cutaneous experiment in dogs, which was of technical grade.

The formulations tested (with percentage concentrations of components) were:

Formulation 1. Tylose HT (4·0), Na_2SO_3 (0·5), 25% ammonia (12·5), *p*-toluenediamine hydrochloride (4·8), resorcinol (0·75), *m*-diaminoanisole sulphate (1·28) and deionized water (76·17); pH 10·1; mixed immediately before application with an equal volume of 6% hydrogen peroxide. This formulation is the same as formulation TRA used by Kinkel & Holzmann (1973).

Formulation 2. *p*-Phenylenediamine (2·0), *p*-toluenediamine hydrochloride (3·0), resorcinol (1·7), *m*-diaminoanisole sulphate (4·0), oleic acid (5·0), isopropanol (3·0), Na_2SO_3 (0·2), concentrated ammonia (6·0) and deionized water (75·1). The oleic acid and isopropanol were mixed with half of the water and half the ammonia and warmed, stirring constantly. *p*-Phenylenediamine, *p*-toluenediamine hydrochloride, resorcinol and *m*-diaminoanisole sulphate were suspended in the rest of the water (warmed to 60°C) and mixed with the remainder of the ammonia. After the addition of Na_2SO_3, this suspension was combined with the previously prepared solution. The mixture was added to an equal volume of 6% hydrogen peroxide immediately before application. This formulation is identical with formulation 7402 of CTFA used by Burnett, Goldenthal, Harris, Wazeter, Strausburg, Kapp & Voekler (1976).

Formulation 3. *p*-Toluenediamine hydrochloride (8·31), 25% ammonia (3·6), deionized water (13·9) and formulation base (74·21). The latter comprised oleic acid (12·0), ammonia (2·9), isopropanol (14·4), alkylamine C_{10}–C_{18} with 2 mol ethylene oxide sulphuric acid semi-ester, sodium salt (5·0), disodium ethylenediaminetetraacetate (0·3), ascorbic acid (0·1), Na_2SO_3 (0·1), perfume (0·4) and water (44·6).

Animals

Specified-pathogen-free Sprague–Dawley rats weighing 90–125 g were allocated at random to groups of nine males and nine females. Three male

and three female beagle dogs weighing 7–11 kg were used and each dog was used in both experiments.

Experimental design

Cutaneous application to the rat. The hair of nine male and nine female rats was clipped in the dorsal region and an area 30 × 30 mm was marked out on the day before the start of the experiment. Before application, the animals were anaesthetized with urethane. Formulation 1 (0·5 g containing 7·5 mg *p*-toluenediamine with a specific activity of 77·8 mCi/g *p*-toluenediamine) was applied to the marked area and left there for 30 min. The dyed area was washed with a commercial shampoo and warm water, rubbed with a towel and dried with a hair-dryer. The animals were kept for 24 hr (calculated from the beginning of application) in metabolism cages. They were then killed, the dyed skin was removed and the carcass was homogenized. The procedure was repeated using Formulation 2 (0·5 g containing 7·5 g *p*-toluenediamine with a specific activity of 22·2 mCi/g *p*-toluenediamine).

Subcutaneous administration to the rat. A 0·4% aqueous solution of *p*-toluenediamine hydrochloride with a specific activity of 12·8 mCi/g *p*-toluenediamine was used. The solution (1 ml containing 3–5 mg *p*-toluenediamine) was administered sc in the dorsal region of each of nine male and nine female rats. These animals were kept in metabolism cages for 5 days. Urine and faeces were collected daily. After this time the animals were killed, the area around the point of injection was removed and the carcass was homogenized.

Oral administration to the rat. A 1·6% aqueous solution of *p*-toluenediamine hydrochloride with a specific activity of 15·8 mCi/g *p*-toluenediamine was used. The solution (1 ml containing 10 mg *p*-toluenediamine) was administered to each of nine male and nine female rats by stomach tube. The animals were kept in metabolism cages for 5 days. Urine and faeces were collected daily. After this time the animals were killed, the gastro-intestinal tract from cardia to anus was removed and both the gut and the rest of the body were separately homogenized.

Cutaneous application to the dog. Formulation 3 (50 ml, containing 1·4 g *p*-toluenediamine with a specific activity of 32·0 mCi/g *p*-toluenediamine) was applied to a marked area of skin 20 × 35 cm on the lateral abdominal region of each of three male and three female tranquillized dogs. After 3 hr the dyed area was washed with a commercial shampoo and warm water, rubbed with a towel and dried with a hair dryer. The animals were kept in metabolism cages for 5 days. The dogs were prevented from licking the treated area by muzzles. Blood samples, urine and faeces were collected at intervals. After 5 days the hair was shaved from the dyed area and its radioactivity was measured, as described below.

Intravenous administration to the dog. The same animals were used for iv administration, 3 wk after the cutaneous application experiment. Each animal was given 0·224 g *p*-toluenediamine hydrochloride (0·14 g *p*-toluenediamine with a specific activity of 15·2 mCi/g *p*-toluenediamine) dissolved in 27·0 ml deionized water. After tranquillization, each animal was infused at a constant rate with 9 ml/hr of the solution, applied by infusion pump and venous catheter via the vena cephalica for 3 hr. The animals were kept in metabolism cages for 5 days. Blood samples, urine and faeces were collected at intervals as in the previous experiment.

Determination of ^{14}C activity

Homogenized portions of 50 or 100 mg of each sample were combusted in a Packard sample oxidizer and the $^{14}CO_2$ was absorbed in Carbosorb and added to the scintillator Permafluor V. The radioactivity was determined in a Packard liquid scintillation counter and corrected for quenching.

RESULTS

The fate of the applied radioactivity 24 hr after cutaneous application of ^{14}C-labelled *p*-toluenediamine to the rat in two hair-dye formulations is shown in Table 1. This shows the amounts absorbed to have been 0·207 ± 0·04 and 0·211 ± 0·02% of the dose from formulations 1 and 2, respectively. Figure 1 shows the percentage of radioactivity excreted over a period of 5 days following sc application of a 0·4% aqueous solution of the labelled compound; at the end of this period 6·9 ± 0·3% of the applied ^{14}C was found in the total body homogenate and 1·7 ± 0·2% remained at the application site. The amount of radioactivity excreted after oral administration of a 1·6% aqueous solution to the rat is also given in Fig. 1. In this experiment the gastro-intestinal tract retained 1·4 ± 0·2% of the applied radioactivity after 5 days and 1·2 ± 0·08% was found in the total-body homogenate.

Following cutaneous application of a hair-dye formulation containing *p*-[^{14}C]toluenediamine to dogs, the amount of radioactivity excreted over 4 days (Fig. 2) totalled 0·092 ± 0·009% and 0·840 ± 0·10% of the administered dose in the urine and faeces, respectively. In these dogs, blood levels of radioactivity reached a peak (925·5 ± 130 pCi/ml) at 6 hr (Fig. 3). After 5 days the hair in the application area retained 1·79 ± 0·2 mCi ^{14}C activity. Peak blood levels of radioactivity were reached in the dog within 2 hr of the start of iv infusion of an aqueous solution of labelled *p*-toluenediamine (Fig. 4). Total amounts of

Table 1. *Distribution of radioactivity after cutaneous application of two formulations containing 7·5 mg* p-[^{14}C]*toluenediamine in the rat*

Location	Radioactivity recovered after 24 hr (% of dose)*	
	Formulation 1	Formulation 2
Excreted in urine	0·14 ± 0·03	0·08 ± 0·01
Excreted in faeces	0·004 ± 0·001	ND
In total-body homogenate	0·063 ± 0·018	0·13 ± 0·01
In application area of skin	5·2 ± 0·6	9·3 ± 0·6
In shampoo water	95·5†	90†

ND = Not detected

*Mean value for eighteen animals ± standard deviation.
†Approximate value.

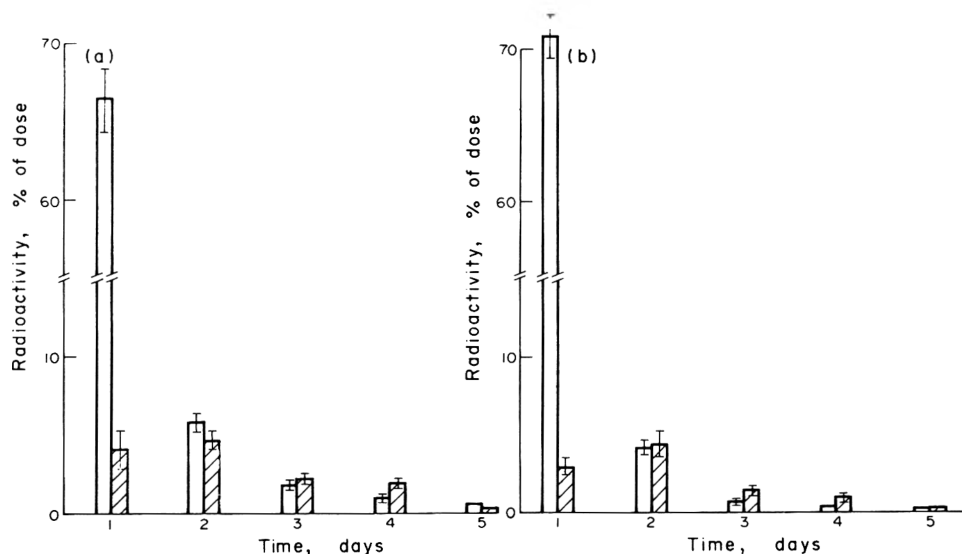

Fig. 1. Excretion of p-toluenediamine (mean ± SD) in the urine (□) and faeces (■) after (a) sc and (b) oral application in aqueous solution to groups of 18 rats.

radioactivity excreted in the urine and faeces were 60 ± 8·0% and 19 ± 4·0% of the administered dose, respectively, with the bulk of the 4-day excretion occurring in the first 24 hr (Fig. 2).

DISCUSSION

In the rat, the degree of absorption of ^{14}C-labelled material was similar for both formulations following cutaneous application. The main difference between the two formulations lay in the amount of radioactivity retained in the application area after washing (approximately 5 and 9% of the dose with formulations 1 and 2, respectively). A further study is planned to determine the exact distribution of the retained material in relation to the morphology of the application site. To calculate the amount of ^{14}C absorbed through the dog skin, the method of Dost (1968) was used. According to this method, the areas under the blood level curves after iv and cutaneous administration (Figs 3 & 4) are determined planimetrically. The ratio of the two areas, taking into account the different ^{14}C-doses applied and the different specific activities, gives an estimate of 0·127% for the cutaneous absorption in this experiment.

Although the extent of absorption could be estimated by comparing the amounts of the radioactivity excreted in urine and faeces, the results would be dis-

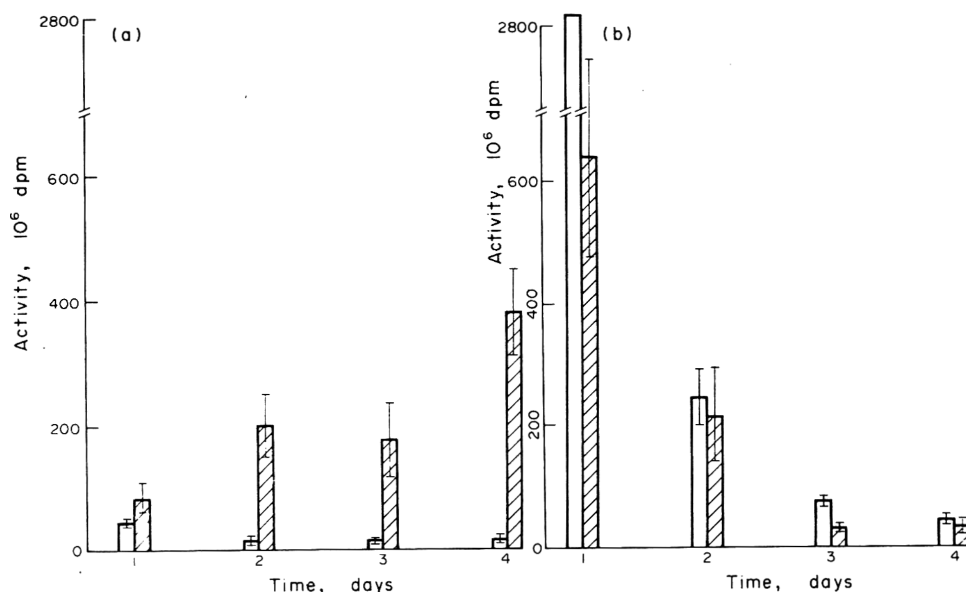

Fig. 2. Excretion of p-toluenediamine (mean ± SD) in the urine (□) and faeces (■) after (a) cutaneous application and (b) iv infusion to groups of six dogs.

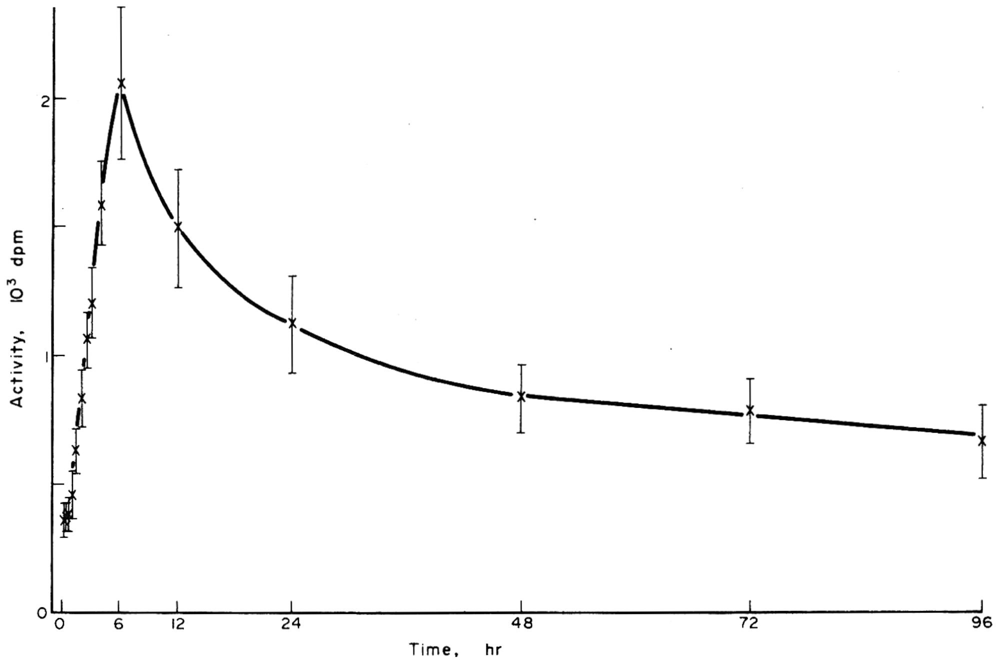

Fig. 3. Levels of *p*-toluenediamine (mean ± SD) in the blood after cutaneous application to six dogs.

torted by contamination of urine and faeces with dyed hair and skin particles containing high levels of radioactivity.

The results reported here are similar to those of Kiese & Rauscher (1968) from analogous experiments in the dog and excretion studies in man. In their experiment the maximum blood level was found after 5–8 hr and the total absorption was 0·3% of the applied amount. These results suggest that a very small amount of this dye constituent is absorbed by human skin in the process of hair dyeing. This amount lies far below the levels used by Isaka (1951) to demonstrate a carcinogenic effect.

In the rat a considerable proportion of the absorbed substance is excreted during the first 24 hr, mainly in the urine, and the situation appears to be

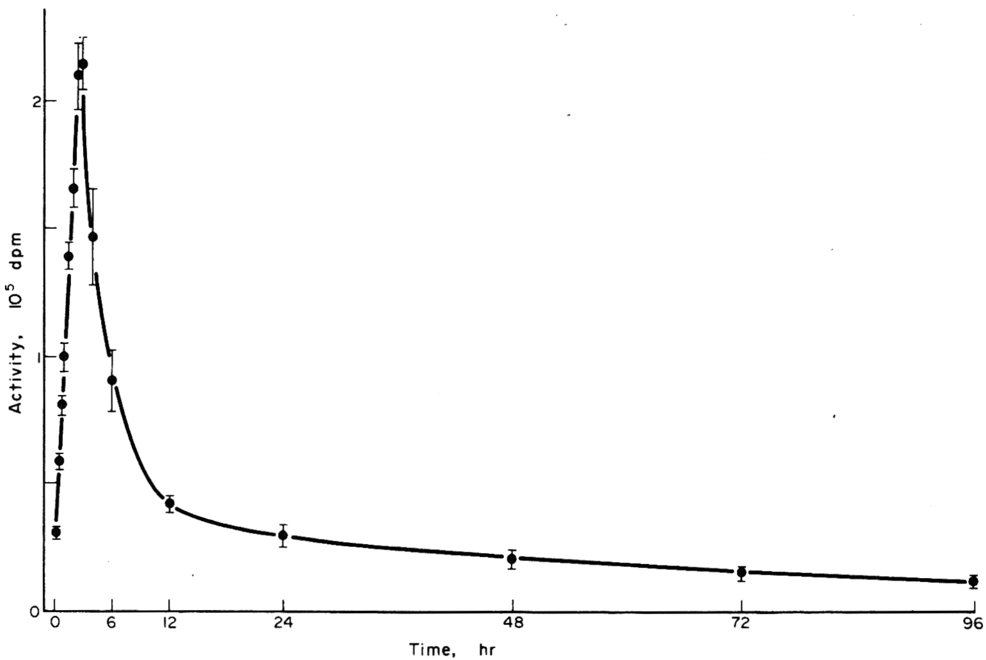

Fig. 4. Levels of *p*-toluenediamine (mean ± SD) in the blood after iv infusion to six dogs.

similar in man (Kiese & Rauscher, 1968). However, in the rat studies, excretion of the absorbed material was not complete after 5 days.

The metabolism of p-toluenediamine was previously investigated by Kiese, Rachor & Rauscher (1968). Their finding that different routes of administration resulted in the same metabolites was important in our calculation of absorption by the dog. It was not thought necessary to repeat these investigations.

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NON-CARCINOGENICITY OF HAIR DYES: LIFETIME PERCUTANEOUS APPLICATIONS IN MICE AND RABBITS

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Abstract—The possible toxicity and carcinogenicity of *p*-amino-*o*-nitrophenol, *p*-phenylenediamine and sodium thioglycollate on mouse and rabbit skin were studied by twice-weekly topical application of these compounds (0·02 ml/animal) in acetone solution. There was no significant decrease in the lifespan of either species. The mice suffered no marked weight loss, and no abnormalities were found in the blood or urine of rabbits. Untreated as well as treated mice presented a large number of tumours, including lymphomas, lung adenomas, liver haemangiomas and ovarian neoplasms; dermal fibromas, but no epidermal tumours, were found in all the treated mice. No statistically significant increase in tumour incidence was found, however, in a comparison of the treated mice with untreated controls, and no neoplasms were observed in the rabbits. In positive controls of both species, large numbers of skin tumours were induced by small doses of 7,12-dimethylbenz[*a*]anthracene, confirming the sensitivity of these models for skin carcinogenesis studies.

INTRODUCTION

Ames, Kammen & Yamasaki (1975), using a bacterial test with *Salmonella typhimurium* as tester strain, recently reported that 89% of commercial oxidative-type hair-dye formulations were mutagenic. Since approximately 30% of the US female population uses hair dye, the hazard could be considerable if these chemicals were mutagenic or carcinogenic in man. However, topical application of hair dyes has failed to yield evidence of carcinogenicity (Burnett, Lanman, Giovacchini, Wolcott, Scala & Keplinger, 1975; Giles & Chung, 1976; Kinkel & Holzmann, 1973), although the interpretation and extrapolation of these data have been subject to criticism (Ames *et al.* 1975).

Sarcomas were reported when the hair-dye component *p*-phenylenediamine (Saruta, Yamaguchi & Nakatomi, 1958) and the closely related *m*-phenylenediamine were injected sc (Saruta, Yamaguchi & Matsuoka, 1962), and hepatocellular carcinomas were found in rats fed another component, *m*-toluenediamine (Ito, Hiasa, Koniski & Marugami, 1969). Formation of *p*-phenylenediamine from dimethylaminoazobenzene has been reported in rats and dogs (Ishidate & Hashimoto, 1959; Stevenson, Dobriner & Rhoads, 1942). Exposure to certain aromatic amines has been shown to cause bladder cancer (Clayson & Cooper, 1970), and the dye *o*-amino-*p*-nitrophenol was found to be mutagenic (Ames *et al.* 1975).

Sodium thioglycollate, a cold-waving agent also included in the present study, differs chemically from the compounds mentioned above but was included because of its similar mode of use.

The purpose of the study was to evaluate the effects of lifetime percutaneous application of the two cyclic amines, *p*-phenylenediamine and *p*-aminonitrophenol, and of sodium thioglycollate in mice and rabbits. The resulting systemic responses, local cutaneous changes and incidence and location of tumours are presented.

EXPERIMENTAL

Chemicals. p-Phenylenediamine (Sigma Chemical Corp., St. Louis, Mo.), *p*-amino-*o*-nitrophenol (Aldrich Chemical Co., Milwaukee, Wis.) and sodium thioglycollate (Fisher Scientific Co., Fair Lawn, N.J.) were used as supplied. 7,12-Dimethylbenz[*a*]anthracene (DMBA: Aldrich Chemical Co.) dissolved in acetone (Fisher Scientific Co.) was used as a positive control. The test compounds were also applied in acetone, in two concentrations selected on the basis of solubility and preliminary determinations of the maximum tolerated dose in mice. The concentrations used were 10 and 5% for *p*-aminonitrophenol and *p*-phenylenediamine and 1 and 2% for sodium thioglycollate.

Mice. Female Swiss mice from the Eppley colony were used when 7 wk old. Littermates were separated and groups of 50 randomly selected animals were housed ten to a plastics cage, on San-i-cel bedding (Paxton Processing Company, Inc., Paxton, Ill.) and given Wayne diet (Allied Mills, Chicago, Ill.) and water *ad lib.* One hundred untreated animals served as controls and 40 DMBA-treated animals were kept as positive controls.

Rabbits. Female rabbits were housed when 8 wk old in pens in groups of five on straw bedding. They were fed a pelleted diet (Wayne, Allied Mills) and given water *ad lib.* The experiment was terminated at wk 85.

Treatment. The chemicals, dissolved in acetone, were applied in a volume of 0·02 ml twice weekly to a 1-cm diameter regularly-shaved area of interscapular skin of the mice and to the inside of the left ear of the rabbits. Animals were checked weekly and all lesions and tumours were recorded. Mice were

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allowed to die spontaneously or were killed when moribund, and rabbits were killed at wk 85. Complete autopsies were performed on all animals, and skin samples, grossly observed tumours and lesions of lungs, livers, kidneys and other tissues from treated and control animals were studied histologically. Formalin-fixed paraffin-embedded specimens were cut and stained with haematoxylin and eosin, and with other stains as appropriate. The statistical significance of the results was evaluated using the methods of Armitage (1971). Haematological, blood chemical and urine analyses were performed on treated rabbits at wk 85. The urine was analysed for specific gravity, pH, glucose, protein, ketone, occult blood, epithelial cells, red and white blood cells and crystals.

RESULTS

Mice

Clinical findings. The average lifespan of the animals was unaffected by treatment (Fig. 1). Unrelated infectious diseases, such as pneumonia and liver infections, occurred in a small number of animals, causing an increased number of deaths. The behaviour of mice in test groups was normal and there were no marked differences between the various groups with respect to body-weight changes and food intake.

Morphological observations. No local changes occurred in treated mice, and no treatment-related epidermal hyperplasia, ulceration or dermatitis were observed. The positive controls showed 67 tumours, including papillomas, kerato-acanthomas and squamous-cell carcinomas, in 38 animals (Table 1).

A number of treated mice had tumours in other organs, but the incidence of these tumours was not statistically different from that of the untreated controls. The most common tumours were lymphomas, mainly of mixed histiocytic and lymphocytic types, with lesions occurring in the lymph nodes, liver, kidneys, intestines, lungs and other organs. The lung adenomas were invariably benign, composed of small cuboidal cells arranged in a glandular pattern. Liver haemangiomas were observed, in addition to haemangiomas of the uterus, subcutis and other organs. The ovarian neoplasms were mostly benign stromal tumours, but, in some, a variable involvement of the granulosa-theca cells was observed. No grossly visible urinary bladder tumours were found, and histological examination revealed no epithelial abnormalities induced by treatment. No epidermal tumours occurred, although mice from all treatment groups showed a few dermatofibromas—benign circumscribed tumours composed of collagen and fibroblasts in the dermis.

Rabbits

Clinical findings. No differences in food intake, weight, behaviour or overall appearance were observed between the groups. The survival rate (Table 2) varied from group to group in a manner unrelated to treatment and dependent on non-specific infectious diseases, such as pneumonia and diarrhoea. No definite signs of toxicity were observed. Haematology and urine analysis of animals treated for 85 wk showed no differences between test animals and untreated controls. Levels of blood glucose, creatinine and urea nitrogen were within normal limits, the serum alkaline phosphatase and serum glutamic–pyruvic transaminase were unchanged and the differential blood count revealed no abnormalities. No abnormal cells were found in the urine, and tests for occult blood, glucose, ketone and proteins were negative.

Morphological observations. No local changes were observed in the ears of rabbits treated with any one

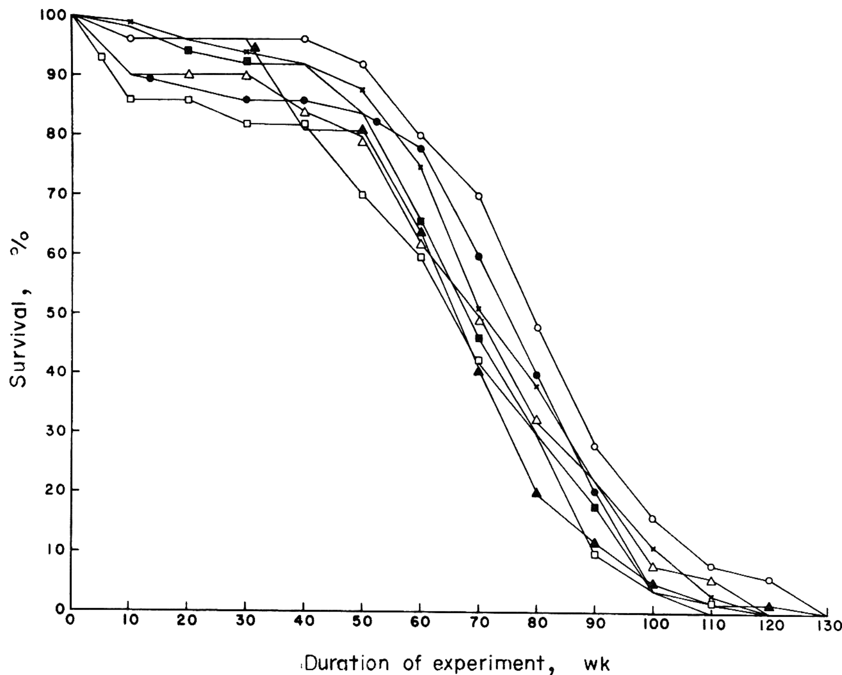

Fig. 1. Survival rates of mice given *p*-aminonitrophenol at 5 (●) and 10% (○), sodium thioglycollate at [1] (■) and 2% (□), and *p*-phenylenediamine at 5 (▲) and 10% (△) compared with those of untreated controls (×).

Table 1. *Number of tumours in mice treated with hair dye components*

Compound	Concn of applied solution (%)	Effective no. of animals	No. of animals with tumours	Total no. of tumours	Tumour types: Lymphoma	Lung adenoma	Liver haemangioma	Ovarian	Subcutaneous fibroma	Others
p-Aminonitrophenol	5	45	23	28	19	5	0	3	1	—
	10	49	24	26	18	4	1	2	1	—
Sodium thioglycollate	1·0	49	19	22	14	3	1	0	1	Sc fibrosarcoma (1) Sc haemangioma (1) Mammary adenoma (1)
	2·0	45	22	24	13	7	2	0	0	Liver leiomyosarcoma (1) Uterine leiomyosarcoma (1)
p-Phenylenediamine	5	44	27	32	16	6	4	4	0	Uterine haemangioma (1)
	10	49	24	34	19	6	1	6	1	Sc haemangioma (1) Mammary adenoma (1)
DMBA (positive control)	0·1	40	38	67	5	0	0	0	0	Papillomas (38) Keratoacanthomas (14) Squamous-cell carcinomas (10)
None (negative control)	—	93	39	46	25	16	1	3	1	—

of the three compounds. In DMBA-treated controls, the neoplastic response was distinct, consisting mainly of proliferating papillomas (15 in five animals), composed of fibrous stroma and squamous epithelium. Other tumours were kerato-acanthomas (five in three animals) and squamous-cell carcinomas (three in two animals). Tumours were not found in other organs in any of the groups.

DISCUSSION

The compounds examined in this study—*p*-phenylenediamine, *p*-aminonitrophenol and sodium thioglycollate—exhibited no toxicity. Changes in the general appearance of the animals, food intake, weight gain and survival rates were unrelated to treatment. In previous studies, topical treatment, which most closely resembles the situation in which these compounds are actually used by man, had failed to show toxic effects (Giles & Chung, 1976). Hexachlorophene caused an increased number of deaths and signs of neurological disturbance in mice after a short period of percutaneous treatment (Stenbäck, 1975), but no such effects were observed in this study.

The Swiss mouse strain is known to be sensitive to percutaneous application of carcinogenic hydrocarbons (Stenbäck, Garcia & Shubik, 1974), and this was confirmed in the DMBA-treated positive control group. However, our studies produced no evidence of carcinogenicity when the three tested cosmetic compounds were applied to mouse skin. Hyperplasia as such, often associated with subsequent skin-tumour formation (Stenbäck, 1969), was not found in these studies.

p-Phenylenediamine, the most common ingredient of permanent hair-colouring preparations, has caused contact sensitization in man (Blohm & Rajka, 1970; Schonning, 1969) and has also been reported to penetrate the epidermis and the hairshaft (Kiese, Rachor & Rauscher, 1968). An allergenic effect accompanied by skin irritation, such as erythema, oedema or scar tissue, as described by Carson, Weinburg & Goldhamer (1965), did not occur, nor was there any increase in cell proliferation.

A possible explanation for the lack of carcinogenicity in skin studies could be the failure of the skin to absorb the compounds. However, some oxidative hair dyes are easily absorbed (Kiese *et al.* 1968) as are many aromatic amines, such as benzidine (Feldman & Maibach, 1970). Kiese & Rauscher (1968) calculated that about 4·6 mg of dye is absorbed by human skin during the hair-dyeing process or is produced *in vivo* from a compound during the preparation of the hair dye and absorbed through the skin when dye is applied to hair and scalp.

Another possibility is that metabolic activation is required for the chemical to be effective. Lack of effect by amines on the skin (Hueper, 1963) is possibly due to the fact that aromatic amines require metabolic conversion to the *N*-hydroxy derivatives to be active (Miller & Miller, 1969).

An increase in bladder-tumour incidence has been reported for male hairdressers (Anthony & Thomas, 1970). Our study failed to show any bladder tumours and these have not been seen in other studies, either experimental (Burnett *et al.* 1975) or epidemiological (Cole, Hoover & Friedell, 1972).

Interpretation of the data concerning systemic tumour response is difficult because of the high incidence of spontaneous tumours, and the limited numbers of animals used. However, because of the widespread use of hair dyes, even a small tumour excess is important when results are applied to the human situation. The relatively short lifespan of the mouse, the impractibility of applying very large doses percutaneously, the limited solubility of the compound and the limited number of animals in this type of experiment make it difficult to achieve maximal exposure to potentially harmful but only weakly toxic or carcinogenic agents.

In relating results from animal tests to the human situation, certain epidemiological aspects must also be considered. Scalp tumours are rare (Burnett *et al.* 1975) and are induced mainly by sunlight (MacDonald & Bubendorf, 1964), a factor which mediates against a possible carcinogenic activity of hair dyes. Moreover, the carcinogenic activity of ultraviolet light on skin is firmly established (Stenbäck *et al.* 1974).

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Table 2. *Survival rate in rabbits treated with hair-dye components*

Compound	Concn of applied solution (%)	No. of rabbits surviving at wk				
		0	20	40	60	80
p-Aminonitrophenol	5	5	5	5	5	4
	10	5	5	5	5	4
Sodium thioglycollate	1·0	5	4	4	3	3
	2·0	5	5	5	4	3
p-Phenylenediamine	5·0	5	5	4	4	4
	10·0	5	4	3	3	1
DMBA (positive control)	0·1	5	4	4	0	0
None (negative control)	—	5	3	2	2	2

Table 3. *Haematology and serum chemistry in rabbits treated with hair-dye components*

Analysis	Standard values*	Concn (%)...	Values following treatment with					
			p-Phenylenediamine		*p*-Aminonitrophenol		Sodium thioglycollate	
			10	5	10	5	2	1
Glucose (mg/100 ml)	164		151	292	342	405	266	264
Urea nitrogen (mg/100 ml)	22		22	19	22	23	29	22
Creatinine (mg/100 ml)	0·9–2		ND	1·5	1·5	1·6	2·0	1·5
AlkPase (units/litre)	6–11		3·7	4·0	5·0	4·5	3·5	5·0
SGPT (units/litre)	19–69		ND	32	18·0	15·5	19·0	18·0
Haemoglobin (g/100 ml)	13·4		10·4	14·9	11·8	11·2	11·0	10·3
Erythrocytes ($10^6/mm^3$)	6·2		4·86	6·58	5·14	4·95	4·90	4·71
Retics (% of erythrocytes)			3·7	4·8	ND	5·0	3·2	4·4
PCV (%)	39		29·6	43·1	33·6	31·6	31·7	30·2
MCV (μm^3)	60		61	66	65	64	65	64
MCH (10^{-12}g)	23		21·4	22·8	23·2	22·5	22·6	22·1
MCHC (%)	35		35·1	34·6	35·5	35·6	34·8	34·2
Leucocytes								
Total ($10^3/mm^3$)	8·1		4·3	8·4	17·8	12·4	5·5	10·9
Differential (% of total)								
Polymorphs	30		9	31	ND	25	24	30
Band neutrophils	—		—	—	ND	—	—	—
Lymphocytes	63		85	63	ND	70	71	64
Monocytes	4		3	6	ND	4	5	6
Eosinophils	1		3	—	ND	1	—	—
Basophils	2		—	—	ND	1	—	—

AlkPase = Alkaline phosphatase SGPT = Serum glutamic-pyruvic transaminase Retics = Reticulocytes
PCV = Packed cell volume MCV = Mean corpuscular volume MCH = Mean corpuscular haemoglobin
MCHC = Mean corpuscular haemoglobin concentration ND = Not determined

*According to Melby & Altman (1974 & 1976).

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SHORT PAPER

ASSESSMENT OF THE ACUTE TOXICITY AND POTENTIAL IRRITANCY OF HAIR DYE CONSTITUENTS

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Summary—The irritant effects on rabbit skin and eye mucosa, and the acute oral toxicity in rats were assessed for twelve compounds which are hair-dye constituents or chemically related aromatic amines, aminophenols, nitro derivatives or aromatic hydroxy derivatives. Five compounds, *p*-aminophenol, *m*-aminophenol, *p*-phenylenediamine, 4-methoxy-*m*-phenylenediamine and toluene-2,5-diamine, were mildly irritant to rabbit skin; the other seven compounds were all non-irritant at the concentrations studied. Positive eye-irritation reactions were elicited by 1-naphthol, but only mild conjunctival reaction followed treatment with the remaining eleven compounds. The acute oral toxicity studies in rats showed a wide variation in LD_{50} values, ranging from 98 mg/kg body weight for *p*-phenylenediamine to 3600 mg/kg body weight for 4-amino-2-hydroxytoluene. The signs of reaction to treatment seen in the rats were similar for each test compound.

Introduction

There has been growing concern about the toxicity of some constituents of hair dyes, mainly aromatic amines and aminophenols and their nitro and hydroxy derivatives. Much of this concern is based upon the results of *in vitro* studies on bacteria (Ames, Kammen & Yamasaki, 1975), and other studies (Burnett, Goldenthal, Harris, Wazeter, Strausburg, Kapp & Voekler, 1976; Hossack & Richardson, 1977) have failed to show positive evidence for carcinogenic or teratogenic effects. The percutaneous absorption of the compounds has been studied in animals (Kiese, Rachor & Rauscher, 1968) and man (Kiese & Rauscher, 1968) and evidence is available of the sensitization potential of some compounds (Adams, 1969; Magnusson & Kligman, 1970). However little or no information is available about the potential primary irritancy or acute toxicity of these compounds.

The experimental investigations reported here were designed to add to the toxicity profile of hair-dye constituents and provide information for the safe manufacture and use of the compounds.

Experimental

Materials

The compounds examined were *p*-phenylenediamine, 4-methoxy-*m*-phenylenediamine, 4-nitro-*o*-phenylenediamine, 2-nitro-*p*-phenylenediamine, *p*-aminophenol, *m*-aminophenol, 2-amino-4-nitrophenol, toluene-2,5-diamine sulphate, resorcinol, 4-chlororesorcinol, 4-amino-2-hydroxytoluene and 1-naphthol. The materials were prepared as aqueous solutions or suspensions in 0·5% aqueous gum tragacanth (Kipton Bros., London, grade 1, BP). Each preparation contained 0·05% anhydrous sodium sulphite (Fisons, Loughborough, SLR grade) and the pH was adjusted to 7·0 ± 0·2 with 0·1 N-NaOH or 0·1 N-HCl.

Methods

Irritant effects in rabbits. The albino rabbits used for the assessment of irritation potential were of the New Zealand White strain.

The procedure for the skin tests was based upon that prescribed for testing primary irritant substances by the Consumer Product Safety Commission of the USA (Code of Federal Regulations, Title 16, Sec. 1500.41). Each of the twelve compounds to be tested was applied as a 2·5% (w/v) preparation to the intact and abraded skin of three animals.

The procedure for the eye tests was also based on that prescribed in the Code of Federal Regulations (Title 16, Sec. 1500.42). Eleven of the twelve compounds were prepared as a 2·5% (w/v) solution or suspension. Eleven groups of three rabbits were used to test these solutions, each of which was instilled into one eye of each of three rabbits. For the twelfth compound, 1-naphthol, 0·5, 1·5, 2·0 and 2·5% (w/v) suspensions were prepared and three rabbits were used to test each concentration. The eyes of all the rabbits were irrigated with 50 ml of lukewarm (37°C) water, 10 sec after instillation of the test material.

Acute oral toxicity in rats. Rats of the CFY strain, in the weight range 90–122 g, were used for the acute oral toxicity tests. The animals were starved overnight before treatment. In a preliminary rangefinding study, the freshly prepared solutions or suspensions were administered to groups of two male and two female rats by oral intubation in a range of dosage volumes, in order to find the approximate median lethal oral dose (LD_{50}). After these preliminary range-finding tests had given a rough approximation of the LD_{50}, larger groups of rats (five males and five females) were used in order to locate the median lethal dose more precisely. A logarithmic dosage interval of 1·6 was used for each material. Rats treated with the vehicle alone served as controls.

During the observation period of 14 days, a record was kept of all deaths and signs of toxicity. All rats

that died were examined macroscopically in an attempt to identify the target organs, and animals surviving to the end of the experiment were similarly examined at that time to detect any possible residual damage. There was no microscopic examination of the various tissues and organ systems. The LD_{50} and its 95% confidence limits were calculated from the mortality data either by the method of Litchfield & Wilcoxon (1949) or by that of Weil (1952).

Results

Irritant effects in rabbits

Skin irritation was observed following treatment with five of the twelve compounds. Toluene-2,5-diamine elicited very slight erythema with slight oedema at the intact site of application and very slight erythema only at the abraded site of one of the three treated rabbits. The reactions ameliorated during the 72-hr observation period. *p*-Phenylenediamine elicited very slight oedema at the abraded sites of two of three rabbits. In one, the reaction ameliorated during the 72-hr observation period. 4-Methoxy-*m*-phenylenediamine elicited very slight oedema at both intact and abraded sites in one rabbit at 24 hr and in another at 72 hr. *p*-Aminophenol elicited similar reactions in one animal at 24 hr and *m*-aminophenol provoked similar reactions at 72 hr. None of the other rabbits treated with these five compounds showed any response to treatment throughout the 72-hr observation period.

The primary irritation index was estimated to be 0·2 for both *m*- and *p*-aminophenol, and 0·3 for toluene-2,5-diamine, *p*-phenylenediamine and 4-methoxy-*m*-phenylenediamine. None of the animals treated with 1-naphthol, resorcinol, 2-amino-4-nitrophenol, 4-amino-2-hydroxytoluene, 4-chlororesorcinol, 2-nitro-*p*-phenylenediamine or 4-nitro-*o*-phenylenediamine showed any response to treatment during the 72-hr observation period.

'Positive' eye irritation reactions, as defined by the test procedure, were produced by only one of the twelve compounds. 1-Naphthol elicited transient corneal opacities (persisting for 1–3 days) in two of three rabbits treated with the 2% suspension and one of three treated with the 2·5% suspension. Transient dulling of the normal corneal lustre (lasting 2 days) was observed in a second rabbit treated with 2·5% 1-naphthol. Considerable conjunctival reaction was seen in two of the three rabbits showing corneal involvement, one treated with 2·5% (score of 2 for swelling) and one treated with 2% 1-naphthol (scores of 2 for swelling and redness). It was noted that two rabbits, one treated with 2·5% and one with 2% 1-naphthol showed only mild conjunctival irritation. Similarly two of three rabbits treated with 1·5% 1-naphthol and one of three treated with 0·5% showed only mild conjunctival reactions. The remaining three rabbits did not show any response to treatment.

Treatment with 2-amino-4-nitrophenol gave rise to a yellow coloration of the cornea 1 hr after instillation, but the cornea was clear by the 24-hr reading, and there was no observable irritant response in any of the animals in this group. None of the three animals treated with 4-methoxy-*m*-phenylenediamine showed any response to treatment, and although occasional transient mild conjunctival inflammation was seen with each of the remaining nine compounds, the reactions did not persist for more than 24 hr.

Acute oral toxicity to rats

The calculated LD_{50} value and 95% confidence limits for each of the 12 compounds are listed in Table 1. Signs of reaction to treatment, observed shortly after dosing with each of the twelve compounds, included lethargy and piloerection. Other reactions elicited by more than one of the test compounds included increased salivation, ataxia, fine body tremors, changes in respiratory rate, diuresis and diarrhoea. Rats given 2-amino-4-nitrophenol or 4-nitro-*o*-phenylenediamine produced orange-stained urine, and those given 2-nitro-*p*-phenylenediamine produced red-stained urine. These signs were not considered to be indicative of any specific target-organ toxicity.

Autopsy of the animals that died as a result of treatment revealed changes which, in many cases, included darkening of the liver and kidneys, darkening or pallor of the spleen, haemorrhage of the lungs and intestines, and injection of the intestinal and mesenteric blood vessels. In addition, two rats treated with *p*-aminophenol showed oedematous swelling of the salivary glands, rats treated with 2-amino-4-nitrophenol showed orange staining of the peritoneal wall, and those given 2-nitro-*p*-phenylenediamine showed red staining of the internal organs. Autopsy of the survivors at the end of each experiment did not reveal any abnormalities indicative of residual systemic effects.

Discussion

The results of the skin tests indicated that *p*-aminophenol, *m*-aminophenol, *p*-phenylenediamine, 4-methoxy-*m*-phenylenediamine and toluene-2,5-diamine may all be considered only mildly irritating to rabbit skin. The other seven compounds tested, 1-naphthol, resorcinol, 2-amino-4-nitrophenol, 4-amino-2-hydroxytoluene, 4-chololoresorcinol, 2-nitro-*p*-phenylenediamine and 4-nitro-*o*-phenylenediamine are essentially non-irritant to rabbit skin.

1-Naphthol is considered to be an eye irritant at concentrations of 2·0 and 2·5% but not at concentrations of 0·5 or 1·5%. On the basis of these observations, the minimum irritant level of 1-naphthol to the rabbit eye is considered to be between 1·5 and 2·0%. Although they elicited occasional minimal conjunctival reaction, the other eleven compounds are considered to be essentially non-irritant to the rabbit eye.

There was considerable variation between the acute toxicities of the twelve compounds, with values ranging from 98 mg/kg bodyweight for *p*-phenylenediamine to 3600 mg/kg for 4-amino-2-hydroxytoluene. The LD_{50} values obtained for *p*-phenylenediamine, *m*-aminophenol, resorcinol, 4-methoxy-*m*-phenylenediamine and 1-naphthol compare favourably with the previously recorded information listed in Table 1. However, discrepancies were noted for 4-nitro-*o*-phenylenediamine, for which the published LD_{50} is 681 mg/kg compared with 2100 mg/kg established in

Table 1. *Results of acute oral toxicity and skin and eye irritation tests on some hair-dye constituents*

Compound	Acute oral toxicity			Irritation studies	
	Concn of test compound (%)	LD_{50} and 95% confidence limits (mg/kg)	Previously recorded* LD_{50} (mg/kg)	Skin irritation (Primary Irritation Index)	Eye irritation†
p-Phenylenediamine	1‡	98 (84–114)	100	0·3	±
Toluene-2,5-diamine	10‡	102 (69–152)	—	0·3	±
4-Chlororesorcinol	10‡	369 (314–433)	—	0	±
Resorcinol	40‡	370 (310–450)	301	0	±
4-Methoxy-*m*-phenylenediamine	10‡	460 (401–522)	515	0·3	−
p-Aminophenol	10§	671 (550–818)	1270	0·2	±
m-Aminophenol	10§	1000 (500–1800)	1660	0·2	±
2-Nitro-*p*-phenylenediamine	40§	1800 (1500–2300)	—	0	±
4-Nitro-*o*-phenylenediamine	36§	2100 (1800–2500)	681	0	±
1-Naphthol	40§	2300 (1700–3300)	2590	0	+
2-Amino-4-nitrophenol	40§	2400 (2000–3000)	—	0	±
4-Amino-2-hydroxytoluene	10§	3600 (3100–4000)	—	0	±

*National Institute for Occupational Safety and Health.
†Categorized as + or − according to whether extrapolation of the result would be expected to give a positive or negative test for eye irritation based on the definition in the Code of Federal Regulations, Test for Eye Irritants (Title 16, Sec. 1500.42).
‡Aqueous solution containing 0·05% Na_2SO_3.
§Suspension in 0·5% aqueous gum tragacanth, containing 0·05% Na_2SO_3.

this study, and *p*-aminophenol, for which the published LD_{50} is 1270 mg/kg compared with 671 mg/kg. No immediate explanation is apparent.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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PRELIMINARY REMARKS ON ALLYL ESTERS

Monographs on several allyl esters appear on pp. 613–621, and the following general point is relevant to all of them.

During human skin testing with allyl esters a delayed type of irritation has occasionally been observed 2 or 3 days after exposure to the ester and has been thought by the investigator to be a case of sensitization. In every case these reactions have been traced to the presence of at least 0·1% free allyl alcohol. While these esters are said to be quite stable, care should be exercised to obtain them as free as possible from this irritant alcohol.

An extensive review of the safety evaluation of allyl esters from the point of view of their use in flavourings has been made by Drake (1975).

Reference

Drake, J. J.-P. (1975). Safety evaluation of allyl esters. *Int. Flavours Fd Add.* **6** (6), 352.

*The most recent of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology* 1976, **14**, no. 6 (pp. 601–633) and Supplement (pp. 659–893).

ALLYL BUTYRATE

Synonyms: Allyl *n*-butyrate; vinyl carbinyl butyrate.
Structure: $CH_2{:}CH{\cdot}CH_2{\cdot}OCO{\cdot}[CH_2]_2{\cdot}CH_3$.
Description and physical properties. A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From allyl alcohol and *n*-butyric acid by direct esterification, or by any other suitable means.
Uses: In public use since the 1950s.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·05	0·04
Maximum	0·1	0·01	0·03	0·4

Status

Allyl butyrate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included allyl butyrate at a level of 1 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health.

Biological data

Acute toxicity. The acute oral LD_{50} value of allyl butyrate in rats was found to be 250 mg/kg, with depression, wet posterior and scrawny appearance for several days and death between 4 hr and 5 days (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The toxicity appears to be related to the presence of the allyl group, and in rats receiving allyl butyrate by stomach tube daily for 4 days, 85 mg/kg/day (one third of the LD_{50}) produced some macroscopic liver lesions, although no deaths ensued (Taylor, Jenner & Jones, 1964). The acute dermal LD_{50} value in rabbits was reported to be 0·53 g/kg (0·35–0·84 g/kg) (Moreno, 1977).

Subacute toxicity. In rats receiving allyl butyrate daily by stomach tube, 90 mg/kg/day given for 18 wk produced growth retardation in males and liver damage (bile-duct proliferation, fibrosis and necrosis); 50 mg/kg/day given for 17 wk produced lung changes (peribronchial lymphocytic infilt-tration) but had no effect on the liver (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

*Irritation**. Allyl butyrate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1977). Tested at 4% in petrolatum it produced low-grade irritation responses after a 48-hr closed-patch test on human subjects (Epstein, 1976).

*Sensitization**. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 28 volunteers. The material (RIFM no. 76-9) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1976).

Metabolism. No cumulative effects would be expected for most butyrates in view of their hydrolysis to materials that are either normally in the diet or readily converted to such materials (Fassett, 1963). In the rat, allyl acetate and allyl alcohol are metabolized to 3-hydroxypropylmercapturic acid, which is excreted in the urine (Clapp, Kaye & Young, 1969).

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*See note on allyl esters, p. 611.

Moreno, O. M. (1977). Report to RIFM, 31 January.
Taylor, J. M., Jenner, P. M. & Jones, W. I. (1964). A comparison of the toxicity of some allyl, propenyl, and propyl compounds in the rat. *Toxic appl. Pharmac.* **6**, 378.

ALLYL CINNAMATE

Synonyms: Allyl β-phenylacrylate; propenyl cinnamate; vinyl carbinyl cinnamate.
Structure: $C_6H_5 \cdot CH{:}CH \cdot OCO \cdot CH_2 \cdot CH{:}CH_2$.
Description and physical properties: A colourless or pale straw-coloured liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of allyl alcohol with cinnamic acid under azeotropic conditions, or by any other suitable means.
Uses: In public use since the 1950s.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·04
Maximum	0·1	0·01	0·02	0·4

Analytical data: Gas chromatogram, RIFM no. 74-16; infra-red curve, RIFM no. 74-16.

Status

Allyl cinnamate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed allyl cinnamate, giving an ADI of 1·25 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 1·52 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964) and the acute dermal LD_{50} value in rabbits as less than 5 g/kg (Levenstein, 1975).

*Irritation**. Allyl cinnamate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Levenstein, 1975), but it was a primary irritant when tested at 4% in petrolatum after a 48-hr closed-patch test on human subjects (Epstein, 1975). Allyl cinnamate produced irritation in concentrations down to 0·25% in the majority of subjects and evoked several borderline irritation reactions at a concentration of 0·1% in a multiple insult irritation test on human subjects (Epstein, 1975).

In tests of acanthogenic activity, application of allyl cinnamate to guinea-pig skin daily for 8–10 days caused very slight histological changes at 5% and severe injury at 40% in acetone, with acanthosis factors of 1·6 and 3·6, respectively, relative to the solvent as 1 (Schaaf, 1961).

*Sensitization**. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 22 volunteers. The material (RIFM no. 74-16) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1975).

Metabolism. Many esters, including benzyl cinnamate, are rapidly hydrolysed *in vivo*. Cinnamic acid is known to conjugate with glycine in the animal body, or it may be converted to benzoic acid (Williams, 1959). In the rabbit, cinnamic acid is almost entirely excreted as hippuric acid, without formation of cinnamoyl glycine (El Masry, Smith & Williams, 1956). In the dog, Quick (1928) observed a large excretion of glucuronide, probably benzoylglucuronide. Dakin (1909) named β-phenyl-β-oxopropionic acid, cinnamoyl glycine and acetophenone as minor metabolites in the dog.

In the rat, allyl compounds are reported to form mercapturic acids, which are excreted in the urine (Clapp, Kaye & Young, 1969).

Capillary permeability. Allyl cinnamate (20% in a sodium alginate base) rubbed on rabbit skin caused some delay (14 min) in the development of a blue tint from trypan blue solution injected iv 15 min later (Pocidalo & Chaslot, 1958).

Anthelmintic activity. In vitro tests with *Rhabditis macrocerca* indicated moderately strong anthelmintic activity, with a concentration of 1·25 mM (0·235 g/litre) causing 50% mortality within 60 min (Cavier & Chaslot, 1956).

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ALLYL CYCLOHEXYLACETATE

Synonym: Allyl cyclohexaneacetate.
Structure: $C_6H_{11}\cdot CH_2\cdot OCO\cdot CH_2\cdot CH{:}CH_2$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of allyl alcohol with cyclohexaneacetic acid under azeotropic conditions, or by any other suitable means.
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·04
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 74-276; infra-red curve, RIFM no. 74-276.

Status

Allyl cyclohexylacetate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included allyl cyclohexylacetate in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 0·90 g/kg (0·56–1·24 g/kg) (Moreno, 1974) and the acute dermal LD_{50} value in rabbits as 1·25 g/kg (0·35–2·15 g/kg) (Moreno, 1974).

*Irritation**. Allyl cyclohexylacetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1974). Tested at 4% in petrolatum it produced a slight irritation after a 48-hr closed-patch test on human subjects (Epstein, 1974).

*Sensitization**. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 23 volunteers. The material (RIFM no. 74-276) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1974).

Metabolism. The hydrolysis of ester linkages in foreign compounds may be catalysed by many different esterases; most of these have a low degree of substrate specificity and they are to be found in all animals and bacteria (Parke, 1968). In the rat, allyl acetate and allyl alcohol are metabolized to 3-hydroxypropylmercapturic acid, which is excreted in the urine (Clapp, Kaye & Young, 1969). In dogs, cyclohexylacetic acid was not aromatized and was probably completely oxidized in the body by β-oxidation (Bernhard, 1937; Williams, 1959).

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*See note on allyl esters, p. 611.

ALLYL HEPTYLATE

Synonyms: Allyl heptanoate; allyl heptoate.
Structure: $CH_2{:}CH \cdot CH_2 \cdot OCO \cdot [CH_2]_5 \cdot CH_3$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of allyl alcohol with heptanoic acid under azeotropic conditions, or by any other suitable means.
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·04
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 74-275; infra-red curve, RIFM no. 74-275.

Status

Allyl heptylate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) included allyl heptylate at a level of 5 ppm (except in chewing gum) in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health.

Biological data

Acute toxicity. The acute oral LD_{50} values in rats, mice and guinea-pigs were reported as 500, 630 and 444 mg/kg, respectively (Hagan, Jenner, Jones, Fitzhugh, Long, Brouwer & Webb, 1965). The acute dermal LD_{50} value in rabbits was reported as 0·81 g/kg (0·44–1·18 g/kg) (Moreno, 1974). Administration of 165 mg/kg (one third of the LD_{50}) by stomach tube daily for 4 days caused death in one of the six rats. Macroscopic liver lesions were observed in all animals; the livers were yellow in colour and approximately half of the hepatic tissue was necrotic (Taylor, Jenner & Jones, 1964).

Subacute and chronic toxicity. Hagan *et al.* (1965) reported that allyl heptylate fed to weanling rats for 18 wk at a dietary level of 10,000 ppm caused severe growth depression in both males and females; the effect was less marked in males at the lower dietary levels and was dose-related. Food efficiency was impaired at 10,000 ppm, but not at 2500 and 1000 ppm. Gross liver enlargement resulted at all three levels, and there were enlarged kidneys in both males and females, enlarged hearts in males given 10,000 ppm, and enlarged testes at 10,000 and 2500 ppm. Microscopic changes consisting of hydropic degeneration of the liver cells in the periportal areas were moderate at 10,000 ppm and less marked at lower levels, showing a dose-related effect. The extent of new bile-duct formation correlated with the degree of hydropic degeneration. Hepatic cell enlargement was noted in some groups.

The same authors reported that all dogs receiving daily oral doses of 75 mg allyl heptylate/kg died within 3–7 months, while those receiving 25 or 5 mg/kg/day were alive after 18 months and showed no gross effects. Administration at 75 mg/kg/day depressed growth and produced a mottled appearance with rough surfaces in the liver and haemorrhagic mucosae in the stomach. Changes observed less constantly were small grey or red cysts of clear aspect in the urinary bladders, and marked congestion in the lungs, digestive tract, kidneys, spleen and lymph nodes. Some organs exhibited terminal haemorrhages. On microscopic examination, all livers showed a slight to moderate fibrosis of the portal areas; this tended to surround hepatic cell lobules of irregular size and shape and was associated with slight to moderate proliferation of the bile-duct epithelium. Slight increases in fat occurred in all but one animal. In the stomach, diffuse haemorrhage and some necrosis of the mucosae were accompanied in some cases by focal submucosal haemorrhages.

In a 2-yr feeding study (Taylor, Hagan & Habermann, 1965), rats received allyl heptylate in combination with seven other fragrance materials and six pesticides at five test levels ranging from the amount occurring in the human daily diet to an amount slightly less than that producing effects in individual toxicity studies. With the exception of growth depression at the highest test level, the findings in the test groups and pesticide control group were similar in all respects to those in the untreated control group.

Irritation.* Allyl heptylate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1974). Tested at 4% in petrolatum it produced a mild irritation (Epstein, 1974) but at 2% in petrolatum it produced no irritation (Kligman, 1975) after similar 48-hr closed-patch tests on human subjects.

Sensitization.* A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 24 volunteers. The material (RIFM no. 74-275) was tested at a concentration of 4% in petrolatum

*See note on allyl esters, p. 611.

and produced no sensitization reactions (Epstein, 1974). The same maximization test was repeated on 25 new volunteers, the material (RIFM no. 74-275) producing no sensitization reactions (Kligman, 1975) when retested at a concentration of 2% in petrolatum.

Metabolism. The hydrolysis of ester linkages in foreign compounds may be catalysed by many different esterases; most of these have a low degree of substrate specificity and they are to be found in all animals and bacteria (Parke, 1968). In the rat, allyl acetate and allyl alcohol are metabolized to 3-hydroxypropylmercapturic acid, which is excreted in the urine (Clapp, Kaye & Young, 1969). Heptanoic acid, like other odd-carbon fatty acids, is converted to glycogen with little or no formation of ketone bodies. The mechanism probably involves β-oxidation with formation of two acetic acid molecules plus one propionic acid molecule (Deuel, 1957).

Micro-organisms. The vapour of allyl heptylate inhibited the *in vitro* growth of the four fungi, *Candida albicans*, *Phoma betae*, *Geotrichum candidum* and *Oospora lactis* (Maruzzella, Chiaramonte & Garofalo, 1961), but did not inhibit *in vitro* growth of three wood-destroying fungi (*Lenzites trabea*, *Polyporus versicolor* and *Lentinus lepideus*) in tests using the filter-paper disc method (Maruzzella, Scrandis, Scrandis & Grabon, 1960). In a 1:500 dilution it did not inhibit *in vitro* growth of *Bacillus subtilis*, *Escherichia coli* or two strains of *Staphylococcus aureus* (Maruzzella & Bramnick, 1961).

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ALLYL PHENYLACETATE

Synonym: 2-Propenyl phenylacetate.
Structure: $C_6H_5 \cdot CH_2 \cdot OCO \cdot CH_2 \cdot CH{:}CH_2$.
Description and physical properties: A colourless slightly viscous liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of allyl alcohol with phenylacetic acid under azeotropic conditions, or by any other suitable means.
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·005	0·1
Maximum	0·15	0·015	0·03	0·25*

Status

Allyl phenylacetate was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included allyl phenylacetate in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 0·65 g/kg (0·51–0·79 g/kg) and the acute dermal LD_{50} value in rabbits as >0·31 g/kg (Moreno, 1976).

Irritation†. Allyl phenylacetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating even when applied at 0·31 g/kg (Moreno, 1976). When it was first tested at 12% in petrolatum, no irritation reactions were reported after a 48-hr closed-patch test on human subjects (Kligman, 1975), but retested at 12% in petrolatum, it produced irritation after a 48-hr closed-patch test (Kligman, 1975). Tested in a 48-hr closed-patch test at 6% in petrolatum, it was a significant irritant in a majority of human subjects, even when it was reduced to 1/4 strength (Epstein, 1976).

Sensitization†. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 75-IFRA-20) was tested at a concentration of 12% in petrolatum and produced sensitization reactions in 12 of the 25 subjects (Kligman, 1975). In a second maximization test, in which the material (RIFM no. 75-IFRA-20) was retested at a concentration of 12% in petrolatum, sensitization reactions were produced in all 25 of the volunteers and the test had to be stopped because of the severity of the reactions on the third induction day (Kligman, 1975).

Because so many reactions to allyl phenylacetate occurred although there had been no similar reactions with the other allyl compounds, it was considered possible that the allyl phenylacetate had an unusually high content of free allyl alcohol. Consequently, the allyl alcohol, which was present at a level of 0·3%, was removed by preparative gas chromatography and a sample that was pure allyl phenylacetate (no. C-37330-R) was obtained. This material was tested by the maximization test (Kligman, 1966; Kligman & Epstein, 1975) in 33 volunteers at a concentration of 3% in petrolatum. It still elicited irritant responses but it induced no sensitization reactions (Epstein, 1976).

Metabolism. In the rat, allyl acetate and allyl alcohol are metabolized to 3-hydroxypropylmercapturic acid, which is excreted in the urine (Clapp, Kaye & Young, 1969).

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*Because of the irritant properties of this material it should probably not be used above 0·25% unless the final product is tested for irritation.
†See note on allyl esters, p. 611.

CALAMUS OIL

Description and physical properties: EOA Spec. no. 101. Typical components of calamus oil are *d*-α-pinene, camphene, cineole, camphor, calamene, calamenol, asaronaldehyde, eugenol, methyleugenol, asarone, calamol, calameone and azulene (Guenther, 1952).
Occurrence: Found in the root of the plant *Acorus calamus* L. (Fam. Araceae).
Preparation: By steam distillation of either the fresh root or the unpeeled dried root of the plant.
Uses: In public use before 1860. Use in fragrances in the USA amounts to less than 2000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·04
Maximum	0·1	0·01	0·02	0·4

Status

The Council of Europe (1974) included calamus in the list of flavouring substances temporarily admitted for use, possibly with a limitation on the active principle in the final product. However, because a 2-yr feeding study carried out in rats by the US Food and Drug Administration indicated that the Jammu variety of calamus oil (Indian) displayed carcinogenic potential, any food or drug within the jurisdiction of the US Federal Food, Drug, and Cosmetic Act containing any form of calamus will be regarded as in violation of the Act (Food and Drug Administration, 1968).

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 777 mg/kg by Jenner, Hagan, Taylor, Cook & Fitzhugh (1964) and as 888 mg/100 g rat (corresponding to 622 g/70-kg man) by von Skramlik (1959), the signs of toxicity in the latter study being convulsions and severe liver and kidney damage, although animals that survived for 3 days recovered completely with no permanent liver or kidney changes. The acute dermal LD_{50} in guinea-pigs was reported as >5 g/kg (Moreno, 1974). The acute LD_{50} of the steam-volatile fraction of the roots and rhizomes of Indian *A. calamus* oil injected ip into rats was found to be 221 mg/kg, and the treatment caused convulsions (Dandiya & Cullumbine, 1959). An oleoresin from rhizomes of Indian *A. calamus* injected ip into mice was toxic in doses of 0·4 and 0·8 g/kg (Dandiya, Baxter & Cullumbine, 1958).

Subacute toxicity. Dietary levels of 2500, 5000 and 10,000 ppm fed to rats for 18 wk depressed growth and caused macroscopic and microscopic liver changes, the effects being less severe at the lower levels; heart changes with minimal to slight myocardial degeneration, characterized by varying degrees of necrosis of muscle fibres, early fibrosis and infiltration with mononuclear cells, were also observed at all levels (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

Irritation. Undiluted calamus oil was not irritating when applied to the backs of hairless mice and swine (Urbach & Forbes, 1974) or to intact or abraded rabbit and guinea-pig skin for 24 hr under occlusion (Moreno, 1974). Tested at 4% in petrolatum it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1974).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 23 volunteers. The material (RIFM no. 74-54) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1974). Bath preparations containing calamus oil have been reported to cause skin erythema (Finkenrath, 1941), and Tulipan (1938) reported dermatitis in hypersensitive individuals. Fifty patients who had reacted to Peruvian balsam, wood tars, colophony and turpentine did not react to calamus oil, and no sensitization reactions were produced by calamus oil in 200 consecutive patients with dermatitis (Rudzki, Grzywa & Bruo, 1976).

Phototoxicity. No phototoxic effects were reported for undiluted calamus oil on hairless mice and swine (Urbach & Forbes, 1974).

Carcinogenicity. In a 2-yr feeding study of the Jammu variety of calamus oil in rats, all the dietary levels tested (500, 1000, 2500 and 5000 ppm) caused growth depression, gross and microscopic (degenerative and regenerative) changes in the liver and damage to the heart. Malignant tumours developed in the duodenum at all dietary levels after wk 59 (Taylor, Jones, Hagan, Gross, Davis & Cook, 1967).

Percutaneous absorption. Calamus oil was not absorbed within 2 hr of application to the intact shaved abdominal skin of the mouse (Meyer & Meyer, 1959). The oil did not aid deep penetration of Rhodamine B into guinea-pig skin (Meyer, 1965).

Micro-organisms. Calamus oil exhibited only slight fungistatic or fungicidal activity against nine of 15 fungi in tests by Maruzzella & Liguori (1958), and slightly inhibited the growth of three wood-destroying fungi (Maruzzella, Scrandis, Scrandis & Grabon, 1960). The oil showed no activity against six bacteria tested by Maruzzella & Sicurella (1960), but Kar & Jain (1971) reported that the essential oil of *A. calamus* showed *in vitro* bactericidal activity against 13 of 15 bacteria tested. In a study of the antifolic-acid activity of anti-epileptic drugs, oil of *A. calamus* repressed the

growth of *Lactobacillus casei* and *Streptococcus faecalis*; the repression was complete at 3200 μg/6 ml but could be reversed at lower concentration (Patil, Chitre & Sheth, 1966).

Pharmacology. European Oleum Calami (from *A. calamus*) demonstrated strong spasmolytic properties when tested on smooth muscle of isolated rat and rabbit intestine, cat trachea, rabbit aorta and rat uterus, and reduced guinea-pig mortality caused by histamine inhalation from 83 to 16% (Maj, Malec & Lastowski, 1966).

The essential oil of Indian *A. calamus* showed anticonvulsant, antiveratrinic and anti-arrhythmic activity (Madan, Arora & Kapila, 1960). Like quinidine, it combated experimental auricular fibrillation in dogs, prolonged the conduction time and refractory period in the electrocardiogram in cats, antagonized the action of dilute solutions of veratridine on frogs (*Rana tigrina*) and had an anticonvulsant action against experimental shock, but was not effective in modifying convulsions produced by metrazole.

At concentrations of 50–100 μg/ml the essential oil of *A. calamus* (European) produced spasmolytic effects, considered to be chiefly myotropic, on isolated smooth muscles from rabbits, guinea-pigs and cats (Shipochliev, 1968a). A 5% emulsion of this oil given iv to cats in a dose of 5–10 mg/kg in physiological saline increased respiratory volume and depressed blood pressure, while guinea-pigs given the oil ip in a dose of 35 mg/kg after preliminary sensitization with egg albumin went into anaphylactic shock (Shipochliev, 1968a). General depression without ataraxia was observed in mice given ip injections of 50 mg/kg as the 5% emulsion, with or without preliminary ip treatment with 150 mg iproniazid phosphate/kg 24 hr earlier. The effect on the central nervous system did not resemble that of reserpine (Shipochliev, 1968b).

An oleoresin from rhizomes of Indian *A. calamus* injected ip into mice at 0·2 g/kg showed only slight sedative activity but considerable potentiation of the sedative activity of sodium pentobarbitone (Dandiya *et al.* 1958).

The steam-volatile fraction of the roots and rhizomes of Indian *A. calamus* reduces the body temperature of mice and prolongs sleeping time when used with pentobarbitone, hexobarbitone and ethanol. It exacerbates the tonic seizures provoked by convulsive doses of metrazole in rats and potentiates the action of reserpine in reducing amphetamine toxicity in mice excited by aggregation. In anaesthetized cats, iv injection of the oil causes a fall in blood pressure which is not prevented by vagal, adrenergic, or ganglionic blockade and does not appear to be due to any nervous mechanism. The oil causes dilation of the blood vessels of the splanchnic area in cats, constricts the blood vessels of the hind legs of frogs, and prevents the action of acetylcholine, histamine, and barium chloride on isolated guinea-pig ileum (Dandiya & Cullumbine, 1959).

Indian acorus oil (50 mg/kg) potentiated pentobarbitone hypnosis in mice; the potentiating action was antagonized by LSD and dibenzyline hydrochloride either separately or combined (Malhotra, Das & Dhalla, 1962). The same dose delayed the rate of disappearance of sodium pentobarbitone from the blood in dogs.

The content of 5-hydroxytryptamine and noradrenaline in the rat brain was depleted by ip injection of Indian acorus oil (100 mg/kg), and it has been suggested that the mechanism of action of the oil may be similar to that of reserpine (Malhotra, Prasad, Dhalla & Das, 1961).

In subacute and acute experiments in mice, rats, cats and rabbits, the essential oil from *A. calamus* (European) exerted a significant sedative and analgesic effect, reduced spontaneous mobility, potentiated the effect of morphine, caused ptosis and reduced body temperature and blood pressure (Maj, Malec & Lastowski, 1964). Enhancement of the effect of barbiturates did not result from changes in barbiturate metabolism. The oil did not exert cataleptic effects nor reduce the toxicity of amphetamine, and its effect was not influenced by iproniazid, as was characteristic of the oil of Asiatic origin. The oil studied was 12–16% as toxic as the Asiatic oil.

Aqueous and alcoholic extracts of the rhizomes of *A. calamus* (European) were administered ip to mice, rats, rabbits, and cats by Maj, Lastowski & Lukowski (1965). The aqueous extract had a sedative effect in mice in doses up to 4 g/kg. The minimum lethal dose of the alcoholic extract in mice was 4 g/kg and the LD_{100} was 8 g/kg. The extracts had no cataleptic or analgesic action in mice in doses up to 1·0 g/kg, did not potentiate the cataleptic action of chlorpromazine and did not show anticonvulsant action in mice and rats. Their effect was not influenced by iproniazid, but they did exhibit hypothermic and hypotensive activity. Only the alcoholic extract potentiated the action of narcotics and diminished the toxicity of amphetamine in mice housed in groups. Both extracts administered in doses of 10–400 mg/kg lowered blood pressure in rabbits and cats by 10–70% (Maj *et al.* 1965).

Indian acorus oil (10–100 mg/kg) administered ip was found to have a sedative–tranquilizing action when administered ip to rats, mice, cats, dogs and monkeys, but vomiting was observed in the latter three species (Dhalla & Bhattacharya, 1968). Acorus oil (10–150 mg) given ip to mice depressed both spontaneous and forced motor activities, with a greater effect on the former. In cats, flexor, patellar and lingomandibular reflexes were inhibited to a varying degree by iv injection of 10–100 mg acorus oil/kg, while the drug did not block the neuromuscular junction or the facilitation of patellar response due to the stimulation of reticular formation. At 0·5 mg/ml, acorus oil inhibited monoamine oxidase activity and stimulated *D*- and *L*-amino acid oxidases *in vitro*. It was suggested that acorus oil might effect its neuropharmacological actions at the spinal cord or subcortical levels of the central nervous system (Dhalla & Bhattacharya, 1968).

Anti-adrenergic activity of Indian acorus oil in the central nervous system was demonstrated by its action in antagonizing the agitational symptoms induced by dexamphetamine in mice, cats, rats, dogs and monkeys, and by the dexamphetamine blockage of its potentiating action on hexobarbitone sleeping time (Bhattacharya, 1968). The oil inhibited the conditioned avoidance response in rats, the effects being more prominent at dose levels inhibiting also the unconditioned response, suggesting some type of ataxic motor impairment (Bhattacharya, 1968).

Medicinal use. Calamus has been used in therapeutic baths for the treatment of weakness and paralysis, including diseases of the bones and muscles such as rickets and poliomyelitis (Birggal, 1969). As the root, oil or extract, it has also been used in drug preparations as a carminative and as a topical counter-irritant (Food and Drug Administration, 1968). Rhizomes of *A. calamus* have been widely used to treat various mental and nervous disorders in India in the Ayurvedic system of medicine (Malhotra *et al.* 1962).

Pest control. Acorus oil has insecticidal and leech-repellent properties which may be synergized by synthetic pine oil (Perti & Agarwal, 1969; Raquibuddowla, Siddiqueullah, Dewan & Haq, 1967; Saxena, Khalsa & Pillai, 1969).

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METHYL ETHYL KETONE

Synonym: 2-Butanone. CAS Registry Number 78-93-3.
Structure: $CH_3 \cdot CO \cdot CH_2 \cdot CH_3$.
Description and physical properties: Merck Index (1976).
Occurrence: Has been reported to occur as an impurity among the products from the dry-distillation of wood and in the oil (extracted with ether) of black tea. It is also present in coffee, cheese, bread, some citrus oils and some other natural products including the grape and raspberry (*Fenaroli's Handbook of Flavor Ingredients*, 1975).
Preparation: By oxidation of *sec*-butanol (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 50,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·08
Maximum	0·1	0·01	0·05	0·3

Analytical data: Gas chromatogram, RIFM no. 74-224; infra-red curve, RIFM no. 74-224.

Status

Methyl ethyl ketone (MEK) was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included MEK in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on this solvent and an extensive monograph was compiled earlier by Browning (1965).

Biological data

Acute toxicity. Acute oral LD_{50} values for MEK were found to be 3·4, 3·6, 3·1 and < 1·0 mg/kg for older adult, young adult, 14-day-old and newborn rats, respectively, by Kimura, Ebert & Dodge (1971), who suggested that the maximum permissible limit for a single oral dose of MEK was 0·0005 ml/kg (1/1000 of the dose giving the first observable gross signs of drug action in young adult rats). Rowe & Wolf (1963) gave the acute oral toxic dose of MEK for rats as 3·3 g/kg, with development of lung irritation and narcosis at massive doses, and the acute dermal LD_{50} for rabbits as > 8 ml/kg. The acute dermal LD_{50} value in rabbits reported by Moreno (1975) was > 5 g/kg. In experimental animals, high concentrations cause narcosis, emphysema of the lungs and congestion of the liver and kidneys, while in liquid form the solvent is highly irritating to the eyes and has been reported to cause oedema of the cornea (Rowe & Wolf, 1963).

The acute ip LD_{50} of MEK for mice was found to be 616 mg/kg (National Institute for Occupational Safety and Health, 1975). When male guinea-pigs were given undiluted MEK in an ip dose of 750, 1500 or 2000 mg/kg, the top dose killed one out of four animals (DiVincenzo & Krasavage, 1974). After 24 hr, elevated levels of serum ornithine carbamoyltransferase (OCT) (indicative of liver damage) were demonstrated in animals given 2000 mg/kg, and lipid accumulation in the liver cells of those given 1500 or 2000 mg/kg. MEK was classified as of low hepatotoxicity, causing elevated serum OCT at doses above 500 mg/kg (DiVincenzo & Krasavage, 1974), a level incorrectly cited elsewhere (National Institute for Occupational Safety and Health, 1975) as the lethal dose for rats.

When administered iv to mice, MEK (0·15 ml of a 10% solution in a physiological medium) caused only temporary narcosis (De Castiglia, Cembal, Fraga de Suarez, Nicolini, Noto & Mitta, 1972).

A recent study was undertaken (Traiger, Bruckner & Cooke, 1975) to determine the effect of 2-butanol (2·2 ml/kg given orally) and MEK (1·87 ml/kg given orally) on the hepatic ultrastructure and microsomal drug-metabolizing activity in the rat. Rats were killed 16, 28 and 40 hr after dosing for the *in vitro* determination of the activities of microsomal acetanilide hydroxylase and aminopyrine *N*-demethylase. A 50–97% increase in acetanilide-hydroxylase activity was found at each of these times after treatment with either compound. Aminopyrine-*N*-demethylase activity was significantly increased in animals treated 40 hr earlier with MEK. Less pronounced increases in *N*-demethylase activity were noted in rats killed 16 or 28 hr after dosing with either agent. Electron microscopic examination of hepatocytes revealed a marginal increase in the amount of smooth endoplasmic reticulum 16 hr after administration of either 2-butanol or MEK and a marked proliferation of this membrane after 40 hr. These results would indicate that the potentiation of CCl_4 hepatotoxicity by 2-butanol or MEK may be related in part to their stimulatory effect on the drug-metabolizing system of the endoplasmic reticulum.

The 48-hr median tolerance level (lethal concentration) for MEK was found to be 5640 mg/litre for bluegill fish (Price, Waggy & Conway, 1974).

Inhalation. A 10% concentration (100,000 ppm) in air caused no deaths among guinea-pigs exposed for a few minutes. Exposure for 1 hr to a 1% concentration (10,000 ppm) had no serious effect,

although irritation of the eyes and nose occurred soon after the start of the exposure and narcosis was observed in 4–5 hr (Patty, Schrenk & Yant, 1935). In animals subjected to lethal doses, marked congestion of internal organs and slight congestion of the brain were observed, the lungs showed emphysema and there was marked congestion in the liver and kidneys; animals that survived exposure to 100,000 ppm for 30 min or more developed corneal opacity, which improved and practically disappeared at the end of 8 days (Patty *et al.* 1935).

Narcosis was evident in guinea-pigs that inhaled 33,000 ppm for 48–90 min, while 10,000 ppm also caused narcosis, with eventual recovery after 240–280 min. Signs of vitamin deficiency were observed in guinea-pigs exposed repeatedly for 12 wk to atmospheric concentrations of 235 ppm MEK (La Belle & Brieger, 1955).

Rats survived inhalation of 2000 ppm MEK for 2 hr, but some deaths occurred after inhalation of 2000 pm for 4 hr or 4000 ppm for 2 hr. The most important effect of the inhalation was narcosis. The predicted effects of daily 8-hr inhalation included some irritation at 250 ppm, marked eye, nose or throat irritation at 500 ppm and definite narcosis (short of dizziness) at 250 ppm (Carpenter, Smyth & Pozzani, 1949; Smyth, 1956).

In rats exposed continuously for 7 days to vapours of methyl *n*-butyl ketone (MBK) at 225 ppm and MEK at 750 ppm separately or combined, hexobarbitone sleeping times were reduced by exposures which included MEK, but not by exposure to MBK alone. *In vitro* hepatic microsomal oxidative- and reductive-enzyme activities were enhanced two- or threefold by exposure to the MBK/MEK combination. This stimulation of enzyme activity may have an important influence on the metabolism of many foreign chemicals, and may help to explain the enhancement of MBK toxicity that occurs with combined MBK/MEK exposures (Hetland, Couri & Abdel-Rahman, 1976).

The threshold limit value for MEK has been set at 200 ppm in air (American Conference of Governmental Industrial Hygienists, 1973). In human studies, exposure to 90–270 ppm MEK vapour for 4 hr was associated with a shortening of time estimates in males and increased variation in females in time-estimation tests of 5, 10 and 30 sec (Nakaaki, 1974). Eye, nose and throat irritation was reported after exposure to 350 ppm for 3–5 min and it was estimated that 200 ppm would be satisfactory for an 8-hr working day (Nelson, Ege, Ross, Woodman & Silverman, 1943). Industrial exposures to MEK vapour have been reported to cause no permanent ill effects at 700 ppm, vomiting and nausea at 500 ppm (attributed to 2-nitropropane) and headaches and throat irritation at 300 ppm, while loss of consciousness resulted from a mixture of 398–561 ppm MEK and 330–496 ppm acetone (Rowe & Wolf, 1963).

Irritation. Methyl ethyl ketone applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1975). Tested at 20% in petrolatum it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1975). Exposure of human forearm skin to MEK for 1 hr/day for 6 successive days resulted in damage to the horny layer, sometimes requiring complete regeneration (Malten, Spruit, Boemaars & de Keizer, 1968). It was earlier shown to cause defatting and partial dehydration of the stratum corneum of hydrated human forearm skin without producing irritation or inflammation (Munies, 1965; Wurster & Munies, 1965). While minor skin contacts encountered in industry did not cause irritation, dermatitis from excessive repeated prolonged skin contact by workers was not uncommon (Rowe & Wolf, 1963). Applied directly to skin affected by poison ivy and poison oak, MEK relieved symptoms and caused drying and whitening of the treated area (Crary, 1975).

Exposure of rabbit skin to MEK for 24 hr caused moderate skin irritation (Rowe & Wolf, 1963). A 50% solution did not aid penetration of Rhodamine B into guinea-pig skin (Meyer, 1965).

Methyl ethyl ketone has a greater capacity to cause oedema of the cornea than has acetone (Larson, Finnegan & Haag, 1956).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 24 volunteers. The material (RIFM no. 74-224) was tested at a concentration of 20% in petrolatum and produced no sensitization reactions (Epstein, 1975).

Metabolism. Ketones are not readily metabolized in the body and may be eliminated unchanged in the expired air and to some extent in the urine. The major metabolic change that ketones undergo is reduction to the corresponding secondary alcohol, which is usually eliminated as the glucuronide. This process has been demonstrated in rabbits, while in dogs, 30–33% of a dose of 0·3–0·5 g MEK/kg was eliminated in the expired air (Williams, 1959). A dose administered iv to mice was rapidly eliminated, primarily by pulmonary ventilation and the remainder as the glucuronic acid ester (De Castiglia *et al.* 1972). Because of its lower solubility in blood, MEK is excreted more rapidly than acetone through the lungs (Rowe & Wolf, 1963). When human subjects were exposed to 300 ppm MEK vapour for 2 hr, MEK was excreted in the expired air (23 ppm) and in the urine (Tada, Nakaaki & Fukabori, 1972). The contamination concentrations in the central nervous and respiratory systems, mucous membranes and kidneys of lunar-module crew members were tabulated for MEK and 300–400 other outgassing products of materials used in the crew compartment (Santoro & Holden, 1971).

Percutaneous absorption was studied by measuring MEK in the expired air of human subjects following application to normal hydrated and dehydrated forearm skin. The MEK was rapidly absorbed, as indicated by its prompt excretion in the expired air. In an 8-hr exposure, a constant level of absorption was reached in 2–5 hr, the absorption rate being increased by hydration of

the stratum corneum. Similar elimination in the expired air was observed following ingestion by human subjects of 375 mg MEK in capsule form (Munies, 1965; Munies & Wurster, 1965; Wurster & Munies, 1965).

In cows fed various rations, MEK has been identified conclusively in the urine, milk and blood, the concentration being highest in the urine (Loney, Bassette & Ward, 1963). The rate of absorption of vapours of MEK through the shells of hens' eggs was reported by Kato, Wantanabe & Sato (1971).

An aerobic soil bacterium, Nocardia, was reported to catabolize MEK by an oxygenase to ethyl acetate, which was then hydrolysed by an acetylesterase to ethanol and acetic acid (Eubanks, 1973). Ilisescu (1971) considered MEK to be easily degradable in biological treatment plants for petrochemical wastes. In modified standard biochemical-oxygen-demand tests, it was found to be 76 and 32% biodegradable in 5 days in fresh water and sea water, respectively, and because of its rapid biodegradation, it is classified by EPA standards as a hazardous and polluting substance (Price *et al.* 1974).

Pharmacology. Anticonvulsant activity was demonstrated in rats given orally a dose of 80·5 mg MEK/kg, which significantly delayed the onset of isonicotinic acid hydrazide-induced convulsions and provided 60% protection against electroshock convulsions, but failed to protect against metrazole convulsions (Kohli, Kishor, Dua & Saxena, 1967). Golubev (1969) reported that 0·25 M-MEK caused contraction of the rabbit pupil, and MEK and other volatile substances isolated from human urine and injected iv into rabbits damaged cerebral and coronary arteries and caused increased capillary permeability, but did not alter the blood-sugar level (Mabuchi, 1969). In dogs, MEK caused vomiting, muscular debility and the formation of large quantities of urinary magnesium ammonium phosphate crystals (Verstraete, van der Stock & Mattheeuws, 1964).

Combined ip administration of MBK and MEK (1:3) to guinea-pigs increased the urinary excretion of the MBK metabolites 2-hexanol and 2,5-hexanedione (Couri, Abdel-Rahman & Hetland, 1976). If the neurotoxic action of MBK is mediated by a metabolite, stimulation of MBK metabolism by simultaneous exposure to MEK may help to explain the marked enhancement of neurotoxicity that is observed with combined MBK/MEK exposures (Hetland *et al.* 1976).

Neurotoxicity. Some workers exposed both to liquid MEK and to its vapours at 300–600 ppm complained of numbness of the fingers and arms (Smith & Mayers, 1944).

In a plastics factory where workers were not using protective gloves and masks and were exposed to tetrahydrofuran–polyester glue and to MEK, both by inhalation of the vapour and by contact with the hands during its use as a cleaning solvent, one worker developed a toxic polyneuritis involving paraesthesia of the fingers, loss of utility of the hand muscles and leucocytosis, which might have been due to MEK or to its combined use with tetrahydrofuran (Viader, Lechevalier & Morin, 1975).

It has been reported (Allen, Mendell, Billmaier, Fontaine & O'Neill, 1975) that in an industry producing plastics-coated and colour-printed fabrics, 86 of 1157 employees were diagnosed as having toxic distal polyneuropathy attributed to exposure to MBK which was present in a solvent mixture with MEK. In 194 employees described as suspected cases on the basis of electrodiagnostic studies, haematological studies showed results that were described as within normal limits. However, an unusual pattern was reported for erythrocyte- and plasma-cholinesterase values obtained for 96 employees with abnormal electrodiagnostic findings. Erythrocyte-acetylcholinesterase (AChE) activities were low (1·37 μmol AChE hydrolysed/mg protein/hr compared with values of 2·03 units obtained for volunteers and neurologically normal patients). Plasma-cholinesterase (butyrocholinesterase or BuChE) values were higher in the case of exposed workers (0·636 mmol BuChE/ml/hr) as compared with normal values (0·253 units). No relationship with the severity of the peripheral neuropathy was found. When a group of ten affected employees returned to work under conditions of reduced solvent exposure, values for erythrocyte AChE and serum BuChE did not differ significantly from values in ten unaffected workers. Average atmospheric levels of 36 ppm MBK and 516 ppm MEK were detected behind the printing machines. A possible synergistic effect with MEK, tetrahydrofuran, trichloroethylene or phthalate plasticizer could not be ruled out in this study. No cases of toxic neuropathy were detected in workers exposed to MEK in another plant where MBK had never been used. Experimental exposures to MEK (not fully described) caused elevated BuChE levels in mice, cats and chickens and depressed erythrocyte cholinesterase levels in mice and rats but not in chickens. Neuropathy has not been found in animals exposed to MEK alone, but combinations of MEK and MBK have shown a substantial synergistic effect in animals (Allen *et al.* 1975).

Rats exposed for 8 hr/day on 5 days/wk for 6 wk to vapours of MBK and MEK (200 and 2000 ppm) developed muscular weakness of the limbs, with some deaths (Duckett, Williams & Francis, 1974). Histological examination of the sciatic nerves of these rats and of rats exposed only to 200 ppm MBK showed axonal hypertrophy, beading and degeneration, associated with widespread perinodal and segmental breakdown of myelin, representing the early changes of neuropathy.

Teratogenicity. Methyl ethyl ketone (1000 or 3000 ppm) inhaled by pregnant rats for 7 hr/day on days 6–15 of gestation was shown to be embryotoxic, foetotoxic and potentially teratogenic (Schwetz, Leong & Gehring, 1974). The concentration of 3000 ppm caused some retardation of foetal development (delayed ossification of sternebrae) and increased gross, skeletal and soft-tissue anomalies, including acaudia, imperforate anus and brachygnathia. No significant maternal toxicity was observed.

Invertebrates. Methyl ethyl ketone does not release alarm behaviour in the honeybee *Apis mellifera* (Boch & Shearer, 1971) or in the ants *Iridomyrmex pruinosus* (Blum, Warter & Traynham, 1966) and *Pogonomyrmex badium* (Blum, Doolittle & Beroza, 1971). It was found to stimulate an increase in physiological age in ticks, *Ixodus persulcatus*, thus increasing their sensitivity to DDT for which MEK is commonly used as a solvent (Uspenskii & Repkina, 1974).

The 24-hr median tolerance level of MEK in the brine shrimp, *Artemia salina*, was found to be 1950 mg/litre (Price *et al.* 1974). It was reported to be an active hatching agent for cysts of the nematode *Heterodera schachtii*, causing maximum hatching (22%) at a concentration of 9 mM (Clarke & Shepherd, 1964).

Micro-organisms. In a study of the inhibitory action of 25 compounds associated with milk against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus lactis* and *Leuconostoc citrovorum*, MEK exhibited some inhibitory action against *Str. lactis* and *L. citrovorum* and sometimes enhanced the growth of *S. typhimurium* (Kulshrestha & Marth, 1974). In other studies, MEK produced only moderate (65%) and temporary inhibition of *E. coli* proliferation at 0·001 M concentration (Együd, 1967; Hata, 1970), and caused little or no inhibition of growth in tests on nine species of bacteria (Kellner & Kober, 1955). It stimulated slightly the germination of uredospores of the wheat-stem rust organism *Puccinia graminis* (French, 1961), was used to inhibit excessive oxidation by the fungus *Fusarium caucasicum* in a patented microbiological oxidation process (Chinoin Gyogyszer es Vegyeszeti Termekek Gyara Rt, 1962), and in a concentration of 0·67 M, was toxic to yeast cells (Lindenberg & Gauchat, 1958). At a concentration of 50 mg/litre in water reservoirs, it inhibited the nitrification process somewhat but did not affect biochemical oxygen demand (Vertebnaya & Mozhaev, 1960).

Cells. At 100 ppm, MEK was moderately toxic *in vitro* to Ehrlich-Landschütz diploid ascites tumour cells (Holmberg & Malmfors, 1974), while at 5000 ppm it provided complete protection against the haemolysis of rat erythrocytes in hypotonic saline solutions (Holmberg, Jakobson & Malmfors, 1974).

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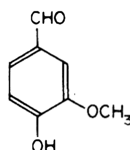
VANILLIN

Synonyms: 4-Hydroxy-3-methoxybenzaldehyde; methyl protocatechuic aldehyde; protocatechualdehyde-3-methylether. CAS Registry No. 121-33-5.
Structure:



Description and physical properties: Merck Index (1976).
Occurrence: Vanillin occurs widely in nature. It has been reported in the essential oil of Java citronella (*Cymbopogon nardus Rendl.*), in benzoin, Peru balsam and clove-bud oil and, chiefly, in vanilla pods (*Vanilla planifolia*, *V. tahitensis* and *V. pompona*). Vanillin is also present in the plants as glucose and vanillin, and another source of vanillin is the waste (liquor) of the wood-pulp industry (*Fenaroli's Handbook of Flavor Ingredients*, 1975).
Preparation: Made synthetically from eugenol or guaiacol. Most vanillin used in fragrances is from the waste (lignin) of the wood pulp industry (Bedoukian, 1967).
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to approximately 250,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·2
Maximum	0·1	0·01	0·03	0·8

Analytical data: Gas chromatogram, RIFM no. 70-10; infra-red curve, RIFM no. 70-10.

Status

Vanillin was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) listed vanillin, giving it an ADI of 10 mg/kg. Both the *Food Chemicals Codex* (1972) and the *United States Pharmacopeia* (1975) have monographs on vanillin and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for vanillin, giving an unconditional ADI of 0–10 mg/kg.

Biological data

Acute toxicity. The minimum lethal dose of vanillin for rabbits was 3·0 g/kg following oral administration as a 5% solution in milk; toxic signs included increased rate of respiration followed by muscular weakness, lachrymation, dyspnoea, collapse and death in coma, without convulsions (Deichmann & Kitzmiller, 1940). The acute oral LD_{50} of vanillin (administered as a 20% solution in propylene glycol) was found to be 1·58 g/kg for rats, with coma, and 1·40 g/kg for guinea-pigs, with depression (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964; Taylor, Jenner & Jones, 1964), and elsewhere the oral LD_{50} for rats was reported as 2·0 g/kg (Hake & Rowe, 1963) and as approximately 2·8 g/kg (Hodge & Downs, 1961).

The lethal dose administered by slow iv infusion to dogs was found to be 1·32 g/kg (Caujolle, Meynier & Moscarella, 1953), and the acute ip LD_{50} values reported were 0·78 g/kg for mice and 1·19 g/kg for guinea-pigs (Caujolle & Meynier, 1954), 1·16 g/kg for rats (Caujolle, Meynier & Farthouat, 1956) and 475 mg/kg for mice (National Institute for Occupational Safety and Health, 1975). Four daily oral doses of 530 mg vanillin/kg given to rats produced no deaths and no macroscopic liver lesions (Taylor *et al.* 1964).

In rats the lethal sc dose was reported as 1·8 g/kg (Deichmann & Kitzmiller, 1940) and the sc LD_{50} as 1·5 g/kg (Binet, 1896), but the sc LD_{50} for vanillin administered as a 4% solution in milk was 2·6 g/kg (Deichmann & Kitzmiller, 1940).

Subacute and chronic toxicity. Intragastric administration of 300 mg vanillin/kg to rats twice weekly for 14 wk produced no adverse effects (Deichmann & Kitzmiller, 1940). Groups of 16 rats were fed diets containing vanillin at levels to provide 20 mg/kg body weight/day for 18 wk without any adverse effects, but 64 mg/kg/day for 10 wk caused growth depression and damage to the myocardium, liver, kidney, lung, spleen and stomach (Deichmann & Kitzmiller, 1940). When ten male and ten female rats were fed diets containing 0·3, 1·0 or 5·0% vanillin for 13 wk, there were growth depression and enlargement of liver, kidney and spleen at the highest level, mild changes at 1·0% and none at 0·3% (Deichmann & Kitzmiller, 1940). In another study (Hake & Rowe, 1963), matched groups of ten male and ten female rats, 4–6 wk old, were maintained for 91 days on diets containing up to 50,000 ppm vanillin, equivalent to about 2500 mg/kg/day. Records of appearance, behaviour,

growth, mortality, terminal body and organ weights, terminal haematological examinations and histological studies, revealed no adverse effects when the diet contained 3000 ppm vanillin, equivalent to as much as 150 mg/kg/day, mild adverse effects followed ingestion of the 10,000-ppm diet and at 50,000 ppm growth was depressed and the liver, kidneys and spleen were enlarged.

Fed to rats at dietary levels of 10,000 ppm for 16 wk, 1000 ppm for 27–28 wk, 20,000 or 50,000 ppm for 1 yr, or 5000, 10,000 or 20,000 ppm for 2 yr, vanillin had no effect on growth or haematology and produced no macroscopic or microscopic changes in the tissues (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

Rats fed for 5 wk on a diet containing a mixture of the maximum permissible amounts of 15 compounds, including vanillin (0·5 g/kg of diet), dyes and insecticides, showed symptoms of intoxication, including decreases in adrenal vitamin C and in liver protein (Sporn & Schöbesch, 1963).

Vanillin injected ip into strain A mice in total doses of 3·6–18·0 g/kg over a period of 24 wk produced no excess of lung tumours and was not considered to be carcinogenic (Stoner, Shimkin, Kniazeff, Weisburger, Weisburger & Gori, 1973).

Irritation. In closed-patch tests on human skin, vanillin caused no primary irritation when tested at concentrations of 20% on 29 normal subjects, of 2% on 30 normal subjects and of 0·4% in 35 subjects with dermatoses (Fujii, Furukawa & Suzuki, 1972).

Sensitization. Maximization tests (Kligman, 1966; Kligman & Epstein, 1975) were carried out on groups of 25 volunteers. The material was tested at concentrations of 2% (Greif, 1967) and 5% (Kligman, 1970) in petrolatum and produced no sensitization reactions.

Vanillin applied undiluted for 48 hr in the standard occluded aluminium-patch test used by the North American Contact Dermatitis Research Group (NACDRG) did not produce any irritation or sensitization in a 62-yr-old subject with a perfume dermatitis (Larsen, 1975). The standard aluminium patch test, designed by the International Contact Dermatitis Research Group to standardize testing for contact dermatitis worldwide, is described extensively by Malten, Nater & van Ketel (1976).

Positive reactions to vanillin were reported in eight out of 142 patients who were already sensitized to balsam of Peru (Mitchell, 1975). In studies of sensitization to balsam of Peru and its components (Hjorth, 1961), vanillin (pure or 10% in vaseline) produced positive patch-test reactions in 21 out of 164 patients sensitive to the balsam. Vanillin was considered to be a secondary allergen, since sensitivity was found only in patients sensitive to vanilla, isoeugenol and coniferyl benzoate. Cross-sensitization to other substituted benzaldehydes was particularly uncommon. Vanillin was found not to be responsible for most cases of sensitivity to natural vanilla.

Vanillin, which appears on the published list of 400 Canadian food additives and is used in artificial flavourings, is known to cause reactions in people previously sensitized to balsam of Peru, benzoin, rosin, benzoic acid, orange peel, cinnamon and clove (Mitchell, 1971).

Metabolism. Early observers noted conversion of vanillin to vanillic acid which was excreted mainly as the free acid, a conjugated ethereal sulphate or glucurovanillic acid (Preusse, 1880). In man, vanillin is broken down by the liver to vanillic acid which is excreted in the urine. Human liver homogenates readily convert vanillin to vanillic acid *in vitro* (Dirscherl & Brisse, 1966). Endogenous vanillic acid production and excretion in man from body catecholamines amounts to <0·5 mg/day, compared with the normal contribution from dietary sources of about 9 mg/day (Dirscherl & Wirtzfeldt, 1964).

In rats, also, vanillin is broken down by the9liver to vanillic acid which is excreted in the urine (Dirscherl & Brisse, 1966). A study of urinary and biliary metabolism of vanillin in rats indicated that oxidative metabolism predominated (65–70%) but that reduction also occurred, with excretion of 19% of the dose as benzyl alcohol derivatives (Strand & Scheline, 1975). When vanillin was fed to rats in doses of 100 mg/kg, most metabolites were excreted in the urine within 24 hr, chiefly as glucuronide and/or sulphate conjugates, although the acids formed were also excreted free and as their glycine conjugates. In 48 hr, 94% of the dose was accounted for, 7% as vanillin, 19% as vanillyl alcohol, 47% as vanillic acid, 10% as vanilloylglycine, 8% as catechol, 2% as 4-methylcatechol, 0·5% as guiacol and 0·6% as 4-methylguaiacol. By investigation of biliary metabolites, prevention of biliary excretion, suppression of intestinal bacteria and inhibition of intestinal β-glucuronidase, it was found that glucuronides of vanillin and its alcohol and acid derivatives are excreted in the bile and that the conjugates are metabolized by the intestinal bacteria to toluene derivatives and decarboxylated products (Strand & Scheline, 1975). Vanillin was found to be reduced to 4-methylcatechol, catechol and 4-methylguaiacol and oxidized to vanillic acid and protocatechuic acid by the intestinal microflora in incubated rat caecal extracts (Scheline, 1972).

When 2-g doses of vanillin were fed to rabbits, products excreted in the urine included 14% conjugated as glucurovanillin and 69% oxidized to vanillic acid, of which two thirds was the free acid and one third was conjugated as the ethereal sulphate and glucuronide (Sammons & Williams, 1941). Glucuronide and sulphate conjugates of vanillin were also identified in cows' milk (Brewington, Parks & Schwartz, 1973).

Metabolism by micro-organisms. Bacteria (21 strains) were able to utilize vanillin, which was oxidized on incubation with chernozem soil to vanillic acid, protocatechuic acid and ring-cleavage products (Kunc, 1971). Vanillin occurring in soil was decomposed by soil micro-organisms, with maximum activity occurring during October to December (Gnittke, Kunze & Steubing, 1971). Vanil-

lin was found to be oxidized by the soil fungus *Actinomyces aureus* to vanillic acid, which was subsequently demethylated and hydroxylated to produce protocatechuic acid, and this was further degraded to succinic acid (Tsai, Chu, Yang & Tsao, 1965). Vanillin was utilized by micro-organisms of both dry mud and sandy sediments of a eutrophic lake (Strzelcyzyk, Donderski & Lewosz, 1972), and vanillin-resistant rhizobia from legume root nodules can utilize vanillin as the sole carbon source (Gupta, Aggarwal & Makkar, 1974). Vanillin was demethoxylated to *p*-hydroxybenzoic acid, alcohol and aldehyde by Saccharomyces yeast in aerobic culture; vanillic acid and vanillyl alcohol were also found but no vanillin remained (Kyowa Fermentation Industry Co. Ltd., 1972).

Insects. Vanillin stimulated feeding behaviour in *Scolytus multistriatus* (the elm bark beetle), with maximum response at a concentration of 0·01 M (Meyer & Norris, 1974), acted as an insect attractant in a patented insecticide composition (Koppers Co., Inc. 1965), and in concentrations of 0·01–0·02 μmol/litre air produced 90% repulsion of the mosquito *Aedes Aegypti* (Burton, 1969).

Invertebrates. Vanillin, which is probably present in the egg-water substance secreted by eggs of *Psammechinus miliaris* (the sea urchin), produced an increase in the fertilization rate but not in sperm respiration (Lybing & Hagström, 1957). The ascaricidal time of vanillin for ascarids of pigs was found to be 3–4 days (Miyama, 1958).

Micro-organisms. Vanillin was reported as having bactericidal activity 5·4 times that of phenol (Führer, 1972). In dilutions of 1:2000 or more it showed antibacterial activity against five bacteria (Katayama & Nagai, 1960), and in a 1:10,000 dilution it exhibited tuberculostatic action against *Mycobacterium tuberculosis* (Jeney & Zsolnai, 1956). Kellner & Kober (1955) reported that vanillin showed low to moderate activity against nine species of bacteria, but Mashimo, Serisawa & Kuroda (1953) found little or no inhibitory activity against four species of bacteria and according to Fiedler & Kaben (1966), vanillin showed no antifungal or antibacterial action against six bacteria and fungi. Rhizobia isolated from legume-root nodules varied in their sensitivity to vanillin (Gupta *et al.* 1974).

Vanillin, a fungitoxic aldehyde found in leaf wound sap, prevented germination of fungal uredospores and teliospores at concentrations of 25–150 μg/ml (Bell, 1970) and, like other lignin destruction products, was found to have fungicidal properties (Telysheva, Sergeeva & Gavare, 1968). At 1 mg/ml it suppressed the growth of *Botrytis cinerea* mould (Ivanova, Davydova & Rubin, 1965), while 1% vanillin stimulated growth of *Coniophora olivacea* but was found to be moderately toxic to *Lenzites trabea* (Rudman, 1963). On the other hand, Zsolnai (1960) reported that vanillin showed no fungicidal activity against seven fungi.

Plants. Vanillin is active as a plant-growth substance, exerting both inhibiting and stimulating effects. These have been studied in Chlorella (Dedonder & Van Sumere, 1971; Dushkova, 1971 & 1973), cereal and leguminous plants (Georgiev & Ivanova, 1972a, b), rice (Uotani, Umezu, Meguro, Tuzimura & Takahashi, 1972), corn (Langdale & Giddens, 1967), Scots pine (Michniewicz & Galoch, 1974), barley (Minchenkova, 1971; Pursakova & Chizhova, 1973; Terent'ev, Tsareva, Semenova & Oskerko, 1974), cotton (Palesiko, Shubert & Ovcharov, 1966), and various crop plants (Helfrich, 1962; Pashkar, Smirnov & Zakharova, 1969). Vanillin was found to be active in retarding the ageing of detached leaves (Karanov, 1969 & 1973; Knypl & Mazurczyk, 1971 & 1972), was toxic to carrot tissue (Goris, 1964) and protected onion cells and barley seedlings against ultraviolet irradiation (Dubrov, 1968).

Pharmacology. Lethal or sublethal doses of vanillin administered orally to anaesthetized rabbits produced sudden depression of the blood pressure and stimulated respiration (Deichmann & Kitzmiller, 1940). Similar results were obtained in dogs (Caujolle *et al.* 1953).

Vanillin produced only a small increase in bile output when administered iv to rats (Rohrbach & Robineau, 1958), and induced some choleretic activity when injected ip into rats in doses of 10–250 mg/kg (Pham-Huu-Chanh, Bettoli-Moulas & Maciotta-Lapoujade, 1968). Injected sc in doses of 1 mg/day for 4 days into immature female rats, it caused a decrease in the ovarian- and an increase in the uterine-weight response to exogenous gonadotropic hormone (Kar, Mundle & Roy, 1960). Vanillin had no effect on the nervous system of fish (Bohinc & Wesley-Hadzija, 1956). In dietary concentrations of 0·05 and 0·1% it had a cariostatic effect in hamsters without impairing growth (Stralfors, 1967).

Vanillin administered as an aerosol had no effect on normally-functioning isolated perfused guinea-pig lungs and did not prevent spontaneous pneumoconstriction (Pham-Huu-Chanh, 1963 & 1964). It did not act as a cross-linking (tanning) agent for corium and aorta, since in 0·15 M solution it did not increase the observed *in vitro* hydrothermal shrinkage temperatures of goat skin and human, bovine and canine aortae (Milch, 1965). It decreased slightly the deformability of dense red cell packs (Jacobs, 1965), and in 1–2 mM concentration produced 50–100% inhibition of collagen-induced platelet aggregation in human blood (Jobin & Tremblay, 1969).

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Fd Cosmet. Toxicol. Vol. 15, pp. 639-640. Pergamon Press 1977. Printed in Great Britain

Review Section

REVIEWS OF RECENT PUBLICATIONS

Evaluation of Certain Food Additives, Twentieth Report of the Joint FAO/WHO Expert Committee on Food Additives. Tech. Rep. Ser. Wld Hlth Org. 1976, no. 599. pp. 32. Sw.fr. 6.00. (Also issued as F.A.O. Fd Nutr. Ser. no. 1, and obtainable in the UK through HMSO).

This report contains a general summary of the conclusions reached at the twentieth FAO/WHO meeting, at which the Committee evaluated a number of food additives for the first time, and also re-evaluated some substances considered at previous meetings. One of the more important substances in the second category was butylated hydroxytoluene (BHT) which had recently been reported to produce an increased incidence of lung adenomas in mice. As *in vivo* and *in vitro* mutagenicity findings were negative, the Committee regarded BHT as unlikely to be carcinogenic, but an adequate carcinogenicity study meeting currently accepted standards was required. The previous requirement for a reproduction study on mixtures of BHT, butylated hydroxyanisole (BHA) and propyl gallate was deleted, and only a multigeneration study on BHA alone was deemed necessary. For propyl gallate, a new long-term mouse study and negative mutagenicity studies were available, but unspecified studies were still in progress in the USSR. It was concluded that the temporary ADIs previously allocated for all three antioxidants should remain unchanged for the present.

The solvent diethylene glycol monoethyl ether was also reviewed. Although the pig was the most sensitive species, no studies extending for longer than 3 months had been conducted, and a 6-month pig study was consequently recommended. Also required before an ADI could be allocated were studies of absorption, distribution, excretion and metabolism, of carcinogenicity in the rat, and of effects on reproduction and possibly teratogenicity. For the caffeine extractant trichloroethylene, a recent report indicating carcinogenicity in the mouse was noted, and the previously accepted use and specification were withdrawn pending the availability of more satisfactorily conducted carcinogenicity studies in two species and information on the levels and nature of residues.

In the case of magnesium silicate, short-term studies were required to determine whether the renal lesions produced by medicinal magnesium trisilicate might also be caused by ingestion of the food-grade material, and in the meantime an ADI was deemed unnecessary. No ADI could be recommended for talc without both a suitable method for detecting asbestos and a long-term study on talc of an acceptable specification.

Specifications and further toxicological data were available on menthol, and the previous ADI of up to 0·2 mg/kg was left unchanged, despite a report suggesting adverse effects in man from ingestion of only ten times this amount. However, the Committee asked for a long-term toxicity and carcinogenicity study in rats, information on the average and maximum likely intakes of menthol, clinical observations on subjects with higher than average intakes, and metabolic studies.

The previous temporary ADI for sucrose esters of fatty acids and sucroglycerides was confirmed, but additional requirements were for studies on the likely sites and degree of hydrolysis of individual sucrose esters, and for a 6-month study in a non-rodent species on a sucrose ester other than the palmitate. Because the esterification process needed to form the glycerol ester of rosin resulted in a very stable bond, long-term and reproduction studies on this material were required before an ADI could be allocated, and further detailed information relating to specification was also requested.

One of the special topics discussed at the meeting was the use of therapeutic and antimicrobial substances in food. Natamycin (pimaricin) was considered unlikely to present problems of microbial resistance and to be of value in preventing the surface growth of moulds, and an ADI of 0·3 mg/kg was allocated.

Another special topic was the kidney lesion produced in rats by high dietary levels of chemically modified starches. It was recommended that further studies should include an examination of changes in urine volume and composition, the effects of minerals in the diet and the presence of parasites, and whether the lesion can be induced in other rat strains or other species. More data on metabolism and specifications were also required.

Sufficient data were still not available to allow consideration of aspartame, and a complete review of cyclamates was deferred. A new monograph was prepared on sodium and potassium nitrites, but recent evidence did not permit the previous temporary status of the ADI to be changed. As calcium hydrogen sulphite is used as a source of sulphur dioxide, it was grouped with sulphur dioxide and sulphites for ADI purposes. Consideration was also given to avian pepsin, glycerol, glycerol diacetate and mineral oil (food grade), for none of which was an ADI considered necessary. In addition new or revised specifications were drawn up for a number of additives, several of which had not been subjected to toxicological evaluation at the meeting.

General priorities for the evaluation of flavouring substances were also discussed by the Committee. It was considered that priority should be given in decreasing order to (1) artificial flavourings unlikely to occur naturally in food, (2) natural materials not nor-

mally consumed as food, their derived products, and the equivalent nature-identical flavourings, (3) herbs and spices, their derived products, and the equivalent nature-identical flavourings, and (4) natural flavouring substances obtained from vegetable and animal products and normally consumed as food, whether processed or not, and their synthetic equivalents. Such a priority list could be developed by an FAO/WHO working group or a group of consultants, and in the meantime substances of lower priority might be tentatively accepted for use in foods if listed as acceptable by the Council of Europe or by national regulatory agencies.

Fd Cosmet. Toxicol. Vol. 15, pp. 641-644. Pergamon Press 1977. Printed in Great Britain

BOOK REVIEWS

Why Additives? The Safety of Foods. Devised and edited by the British Nutrition Foundation. Forbes Publications, London, 1977. pp. xvi + 75. £1.50.

The "chemicals in our food" have faced considerable adverse publicity over the last few years. This booklet, written for the layman, presents the justifications for the use of food additives and should help to redress the balance.

Short essays by 15 authorities in the field briefly describe the mechanisms of food-additive control in the UK and EEC, the functions of the major classes of direct food additives and the biological effects of such additives. Adventitious additives derived from animal feedstuffs and pesticide application are also discussed, although surprisingly those from food packaging have been omitted. Although written in 1968, the introductory essay by the late Sir Alastair Frazer, entitled "Some problems of the evaluation of safety-in-use of a food additive", has lost little of its relevance. Points that are deservedly emphasized are the chemical nature of food as well as of food additives, and the much poorer food supply we would have if additives were not used. Attention is also drawn to the lengthy process of assessment of both technological need and safety that an additive must undergo before its use is permitted.

Apparently the authors made no attempt to avoid trespassing on each other's subjects, and this has led to a certain amount of repetition (for example in connexion with the hazards of nitrosamine formation and the use of the hundredfold safety factor in extrapolating animal data to man). On a more specific point, the statement in the chapter on the biological effects of food additives (by Drs A. Pottage and W. S. Nimmo of Edinburgh Royal Infirmary) that "there may be a threshold level below which [liver enzyme] induction [by BHA, BHT and certain pesticides] does not occur" seems unduly pessimistic, since there is ample experimental evidence that enzyme induction occurs only at levels well above those normally ingested by man. The added implication that dietary levels of these compounds may in consequence lead to vitamin D deficiency could also cause unwarranted anxiety in the general public; this effect has only been observed in connexion with prolonged high-dose administration of drugs such as phenobarbitone and diphenylhydantoin.

However, as a whole, the book presents a well-balanced review of its subject and should help to allay unnecessary fears rather than raise them.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 62. Edited by F. A. Gunther. Springer-Verlag, New York, 1976. pp. viii + 181. DM 41.00.

Having completed its fifteenth year, this series shows no sign of flagging and no reduction in the variety of its subject matter within its now broad terms of reference.

Volume 62 is concerned principally with "worker reentry safety", reproducing nine reports that were originally presented at a symposium forming part of the 167th (1974) National Meeting of the American Chemical Society. These papers throw considerable light on the problems of resuming work in areas where crops have been sprayed, particularly with organophosphorus pesticides, and on the steps that have been or may be taken to control and monitor worker exposure and lay down suitable safety intervals during which substantial contact with treated foliage must be avoided. This is a problem of particular concern to the citrus growers of California, but its consideration in this series of papers is likely to be of general interest to many agricultural communities outside the fruit-growing belt of North America.

A wide-ranging review of selenium in the environment occupies one of the two remaining chapters in this volume. Few elements can have evoked more controversy during the past decade or so, with allegations of carcinogenicity and demonstrations of acute toxicity competing with evidence of its essential role in animal nutrition. It is probable that it is also an essential element for man, but since human diets are generally well supplied with it the problems of selenium deficiency do not arise in this context. The literature on selenium is already enormous, but this concise and well-organized review is a useful addition to a complex subject.

Finally, the fate and toxicity of two herbicides are considered, particularly from the standpoint of their potential environmental effects. Both diquat, a quick-acting contact herbicide, and endothall, a possibly less familar selective herbicide, are widely used for the control of aquatic weeds, and this review pays particular attention, therefore, to the influence these compounds may have on aquatic ecosystems.

Datensammlung zur Toxikologie der Herbizide. Deutsche Forschungsgemeinschaft—Kommission für Pflanzenschutz-, Pflanzenbehandlungs- und Vorratsschutzmittel. Series 2. Verlag Chemie GmbH, Weinheim, 1976. pp. 176. DM 98.00.

A second group of monographs on the toxicology of herbicides has been issued under the auspices of the Deutsche Forschungsgemeinschaft commission concerned with agents for plant protection and treatment. The working group responsible for this project issued its first set of monographs in 1974 (*Cited in F.C.T.* 1975, **13**, 460), and publication in loose-leaf

form permits the combination of these two sets of material, and any that may be produced in the future, into a single well-ordered unit.

The original format has been maintained in the more recent monographs. Details of identification, physical properties, range of application and general functional characteristics are followed by toxicity data derived from animal and *in vitro* studies and from cases of exposure of man and domestic, farm and wild animals, by metabolic data relating to both animals and plants, and by information on breakdown in soils and water, on interactions with other materials and on appropriate therapy for cases of poisoning. Finally an overall toxicological assessment is undertaken and a list of relevant references is presented.

The herbicides covered in the current series are cycluron, dalapon, dicamba, dichlobenil, dinoseb and dinoseb acetate, DNOC, linuron, monalide, monolinuron, monuron, sodium chlorate, pentanochlor, prometryne, propazine and simazine. An appendix identifies trade-names and manufacturers of commercial preparations containing these compounds and some of the herbicides considered previously.

This is an ambitious and useful project which must command attention not only on account of the well-known and respected names that constitute the working group but also because of the way the group has tackled its task.

Handbook of Industrial Toxicology. By E. R. Plunkett. Heyden & Son Ltd., London, 1976. pp. 552. £17.50.

Although this book covers a modest 1500 industrial chemicals compared with the 13,000 or so considered by Dr. Irving Sax (*Cited in F.C.T.* 1976, **14**, 202), there are compensations. The main section of Dr. Plunkett's book is well laid out, each compound being considered under the headings: material description, type of exposure, toxicity (including mode of action), signs and symptoms, tests and treatment, and preventive measures. The most useful information is contained in the treatment section, and while this is very brief, often only a sentence or so, it could conceivably justify the publishers' contention that the book belongs alongside a factory's first-aid box. The author is to be commended for providing references to general review articles on many of the materials listed, but it is unfortunate that this coverage could not have been more extensive. Surprisingly, there is no list of contents; the reader is left to find his own way through a short bibliography as well as a number of appendices covering a cyanide-poison antidote kit and treatment of methaemoglobinaemia, the preparation of a fat biopsy, details of the TLVs for chemical substances in workroom air adopted for 1974 by the American Conference of Governmental Industrial Hygienists, and the 1971 ILO U/C international classification of radiographs of pneumoconioses.

This publication, unlike 'Sax', does not suffer from excessive use of the term "suspected carcinogen", but at times the reverse criticism is due. For example, the infamous dioxin and asbestos are not considered to merit the description "highly toxic", a term given to such compounds as formaldehyde and hydroquinone. In general, however, the well-organized descriptive listing of hazards will provide a useful background for laboratory and factory workers concerned with the handling of chemicals, although it has little to offer the industrial toxicologist with more quantitative needs.

Mammalian Cell Membranes. Vol. 1. General Concepts. Edited by G. A. Jamieson and D. M. Robinson. Butterworths, London, 1976. pp. x + 276. £18.00.

Mammalian Cell Membranes. Vol. 2. The Diversity of Membranes. Edited by G. A. Jamieson and D. M. Robinson. Butterworths, London, 1977. pp. xi + 364. £18.00.

Mammalian Cell Membranes. Vol. 3. Surface Membranes of Specific Cell Types. Edited by G. A. Jamieson and D. M. Robinson. Butterworths, London. 1977. pp. x + 276. £18.00.

The investigation of membrane structure and function is an ever-growing field and a descriptive analysis of the current state of knowledge constitutes a task of considerable magnitude. Equally it presents the reviewer with an unenviable task.

The editors of this series state that the aim of the treatise has been to review the state of the membrane field in the light of current knowledge without involving the reader in extensive collections of raw data. In this they have been reasonably successful, and most authors have considered the basic concepts of their particular expertise in the light of recently published data. Obviously in a venture of this kind and magnitude, points of controversy arise, but these should stimulate thought on the part of the reader. Unfortunately there is also some repetition of data, and one feels that it might have been better to deal with certain topics in depth in one particular section rather than to offer some coverage in a number of different chapters. However, this would probably have restricted the range of viewpoints presented.

The first three volumes of this five-volume work deal in turn with the physical and chemical studies that have formed the basis of current knowledge of membrane structure and function, with the specific types of intracellular and extracellular membranes found in mammalian tissues and with the surface membranes of certain types of cells, such as erythrocytes, tumour cells, nerve cells, spermatozoa and ova, which have been studied in reasonable detail. Little consideration is given in these three volumes to the biochemical and physiological aspects of membranes, but this deficiency should be rectified by the next instalment, which is destined to consider membrane and cellular function from the biochemical, physiological and ultrastructural standpoints. The fifth volume in the series will examine the response of the cell surface membrane (plasma membrane) to various factors in the extracellular environment.

In summary, the three volumes currently available provide an insight into the field of mammalian membranology which remains of considerable value alongside previously published membrane reviews. Further volumes in the series may enhance its value as a constructive source of knowledge.

Histological Techniques. By M. Gabe. Masson, Paris, and Springer-Verlag, Berlin, 1976. pp. xxiii + 1106. DM 120.80.

"La technique n'est rien; la manière de l'appliquer est tout."

(A. Branca, 1924)

Towards 1800, the Parisian anatomist Xavier Bichat propounded the notion of a tissue and thus laid the foundations of histology, a science devoted to the morphological study of tissues and their constitutive elements.

At that time, thin hand-cut sections stained with natural dyes were examined under a simple monocular lens. Progress has accelerated rapidly since then with the introduction of coal-tar dyes and improvements in microscope optics. The stains developed by such famous men as Ehrlich, Weigert, Perls and Feulgen are still widely used today, but other techniques of that era were overtaken by subsequent developments in microscopy and dyestuffs. Fluorescence, interference and phase-contrast microscopic techniques became available, together with numerous dyes seconded from the textile industry. This classical period of histology has now passed and interest is concentrated on specificity, purity and quantitation. Mucoproteins are now glycosaminoglycans—no longer stained empirically by Southgate's mucicarmine method but with alcian blue at pH 1·0 or 2·5 to demonstrate sulphated or carboxylated groups respectively.

To attempt to cover such a diverse period of histological development is an unenviable task. Dr. Gabe has encompassed this in one large volume divided into five parts for ease of reference. Part I covers the general principles involved in the handling of tissues, vital staining, embedding techniques and cutting, while the general methods dealt with in Part II include the majority of the 'routine' stains. The third and largest section, of over 400 pages, is concerned with histochemical techniques for the demonstration of metals, carbohydrates, lipids, proteins and enzymes. Part IV describes the established methods for studying the nucleus, Golgi apparatus, mitochondria and secretory granules, and the final part is a large section of 300 pages describing techniques for studying the majority of mammalian tissues, including the central nervous system, as well as techniques applicable to such tissues as the digestive glands of invertebrates and the swim-bladder of fish.

The appearance of the book is nostalgic; the variations in type face, the layout and the paper itself remind one of a bygone era. The translation from the French is excellent, phrases such as "....a chemical compound which has been placed on the market by good suppliers of requirements for microscopy...." being the exception. This book should be judged not by its omissions, which are numerous because of its broad coverage, but by the great diversity of its inclusions. Many of the techniques will be unfamiliar to histologists in the UK but are well known to colleagues in Mainland Europe. The index is just adequate—only with prolonged browsing can one find such rarities as the method for "bulk staining of Corti's organ"—and familiarity undoubtedly increases the book's value.

This book, as the foreword points out, "is based on more than a quarter of a century of reading, reflexion and especially of the daily practice of histological technique. It is addressed to those who in turn wish to experience the harsh joys of encountering a subject that is difficult but of inexhaustible wealth." While it may not satisfy the pathologist addicted to modern quantitative techniques, it is a must for the traditionalist who still gets pleasure from a good 'H & E'.

Mass Spectrometry. Vol. 4. Senior Reporter R. A. W. Johnstone. The Chemical Society, London, 1977. pp. xii + 357. £22.50.

This volume provides a valuable source of reference to many of the more significant papers on mass spectrometry published between July 1974 and June 1976. Following the format of previous volumes, the obligatory coverage of the theoretical aspects of the subject is quickly followed by reviews of the extensive and ever-expanding literature on application of the numerous forms of the technique.

Each of the 12 chapters has a different author and between 60 and 810 references, so inevitably there is some overlapping between contributions. New chapters on "Trends in instrumentation" and "Field ionization and field desorption" reflect the proliferation of 'hardware' which is making mass spectrometry such a growth industry. Clearly a great deal of useful and reasonably up-to-date information is available in these pages, but for a book of this kind the promise of a cumulative index in volume 5 hardly compensates for the absence of a subject index in this and previous volumes.

BOOKS RECEIVED FOR REVIEW

Immunology of the Gut. CIBA Foundation Symposium 46. Edited by R. Porter and J. Knight. Excerpta Medica, Amsterdam, 1977. pp. viii + 376. $28.00.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 65. Edited by F. A. Gunther. Springer-Verlag, New York, 1976. pp. viii + 103. DM 36.20.

Trace Elements in Human Health and Disease. Vol. II. Essential and Toxic Elements. Edited A. S. Prasad and D. Oberleas. Academic Press, Inc., (London) Ltd., London, 1976. pp. xviii + 525. £22.90.

Lecture Notes in Biomathematics. Vol. 11. Mathematical Models in Medicine. Workshop, Mainz, March 1976. Edited by J. Berger, W. Bühler, R. Repges and P. Tautu. Springer-Verlag, Berlin, 1976. pp. xii + 281. DM 28.00.

Current Topics in Pathology. Vol. 63. Pathology of the Gastro-intestinal Tract. Edited by B. C. Morson. Springer-Verlag, Berlin, 1976. pp. 353. DM 96.00.

Caffeine and Chromosomes. By B. A. Kihlman. Elsevier Scientific Publishing Company, Amsterdam, 1977. pp. xviii + 504. $63.25.

Handbook of Experimental Pharmacology. Vol. XVI. Experimental Production of Diseases. Part 5. Liver. Edited by O. Eichler. Springer-Verlag, Berlin, 1976. pp. xiii + 377. DM 192.00.

Pesticide Manual. Basic Information on the Chemicals used as Active Components of Pesticides. 5th Ed. Edited by H. Martin and C. R. Worthing. Issued by the British Crop Protection Council, 1977. pp. ii + 593. £15.00.

1975 Evaluations of Some Pesticide Residues in Food. The Monographs. Joint Meeting of the FAO Working Party of Experts and the WHO Expert Committee on Pesticide Residues, Geneva, 24 November–3 December 1975. AGP: 1975/M/13; WHO Pesticide Residue Ser. no. 5, Geneva, 1976. pp. viii + 409. Sw.fr. 32.00.

Wholesomeness of Irradiated Food. Report of a Joint FAO/IAEA/WHO Expert Committee. Tech. Rep. Ser. Wld Hlth Org. 1977, **604.** pp. 44. Sw.fr. 6.00.

Clinical Toxicology. Proceedings of the European Society of Toxicology. Vol. XVIII. Edited by W. A. M. Duncan and B. J. Leonard. Excerpta Medica, Amsterdam, 1977. pp. ix + 348. DM 87.50.

Metals and Metabolism. By D. A. Phipps. Clarendon Press: Oxford University Press, London, 1976. pp. viii + 134. £5.00.

Radiation Chemistry of Major Food Components. Its Relevance to the Assessment of the Wholesomeness of Irradiated Foods. Edited by P. S. Elias and A. J. Cohen. Elsevier Scientific Publishing Company, Amsterdam, 1977. pp. xii + 220. Dfl. 63.00.

Fd Cosmet. Toxicol. Vol. 15, pp. 645–648. Pergamon Press 1977. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

THE METABOLISM AND TOXICITY OF SAFROLE AND ESTRAGOLE

The naturally-occurring flavouring safrole (4-allyl-1,2-(methylenedioxy)benzene) is a weak liver carcinogen in rats and mice, male mice being more susceptible than females. After oral or ip administration to various species, up to 33% of the dose was excreted in the urine as the glucuronide conjugate of 1'-hydroxysafrole (OHS), which was considerably more hepatocarcinogenic than safrole (*Cited in F.C.T.* 1974, **12**, 417). However, the ultimate carcinogenic metabolite of safrole may be 1'-acetoxysafrole (AcOS), which on injection into newborn mice produced a liver-tumour incidence similar to that induced by OHS but which, unlike the latter compound, reacted with nucleosides and methionine. This showed it to be an electrophile, as are most chemical carcinogens. Further work revealed that neither safrole nor OHS was mutagenic in the Ames Salmonella/microsome test, whereas AcOS gave positive results (McCann *et al. Proc. natn. Acad. Sci. U.S.A.* 1975, **72**, 5135).

AcOS has not yet been identified as a metabolite of safrole, and a number of other compounds have emerged as possible candidates for the role of ultimate carcinogenic metabolite. When Stillwell *et al* (*Drug Metab. Dispos.* 1974, **2**, 489) gave safrole by ip injection to rats and guinea-pigs in single doses of 125 and 50 mg/kg, respectively, the major urinary metabolites were 1,2-dihydroxy-4-allylbenzene, OHS (1,2-methylenedioxy-4-(1'-hydroxyallyl)benzene), 1,2-methylenedioxy-4-(2',3'-dihydroxypropyl)benzene, 1,2-dihydroxy-4-(2',3'-dihydroxypropyl)benzene, 2-hydroxy-3-(3,4-methylenedioxyphenyl)propionic acid and 3,4-methylenedioxybenzoylglycine. In the conversion of the allyl side chain of safrole to a 2',3'-dihydroxypropyl side chain in the third metabolite (a diol), safrole epoxide was suspected as an intermediate, and ip administration of this epoxide to both species resulted in the excretion not only of the expected diol, but also of the fourth and fifth metabolites listed above and of 2-hydroxy-3-(3,4-dihydroxyphenyl)propionic acid. A small amount of a triol, 1,2-methylenedioxy-4-(1',2',3'-trihydroxypropyl)benzene, was found only in rat urine. Unchanged safrole epoxide persisted in the urine of both species indicating that it was sufficiently stable to circulate in the blood and be excreted in the urine. In view of its electrophilic reactivity with nucleosides (Wislocki *et al. Cancer Res.* 1976, **36**, 1686), this epoxide qualifies as a potential ultimate carcinogenic metabolite of safrole.

Other reactive metabolites, namely safrole 1'-sulphate and 1'-hydroxysafrole-2',3'-oxide (OHS-O), were subsequently shown to be formed from OHS by rat- and mouse-liver enzyme systems *in vitro* (Wislocki *et al. loc. cit.*). When [2',3'-^{3}H]OHS was administered to rats, mice and hamsters in an ip dose of 100 mg/kg, the specific activities of the liver macromolecules 16 hours later were generally in the order ribosomal RNA = protein > DNA, and the nucleosides contained very little, if any, ^{3}H. Alkaline digestion of the liver homogenates or solvent-extracted liver protein liberated 0·1–0·3% of the ^{3}H in the form of 3'-methylmercaptoisosafrole, previously identified as a reaction product of AcOS and methionine (*Cited in F.C.T.* 1974, **12**, 417). However, attempts to demonstrate the formation of AcOS from OHS by incubation with microsomes, mitochondria or cytosol from rat or mouse liver were unsuccessful. The sulphate, rather than the acetate, appeared to be the principal ester formed from OHS, since in rat- and mouse-liver cytosols [2',3'-^{3}H]OHS became bound to ribosomal RNA in a reaction which was dependent on 3'-phosphoadenosine 5'-phosphosulphate. This reaction took place at a rate of 0·1 μmol/g liver/hour in the livers from rats and female mice, although in livers from male mice the rate was considerably slower. The reactive metabolite OHS-O was also formed from OHS when this was incubated with liver microsomes fortified with an NADPH-generating system. Its rate of formation was 0·05–0·3 μmol/g liver/20 minutes, the highest values being obtained in the livers from mice of both sexes, and the lowest in female rat livers. However, no evidence could be found for the formation of another potential metabolite, 1'-oxosafrole. OHS-O was shown to react electrophilically with the nucleosides guanosine, uridine, adenosine, thymidine and cytidine, to an extent varying from 10% with guanosine to 3·2% with cytosine. Relative potencies of this and other potential safrole metabolites in this respect were in the order 1'-oxosafrole > AcOS > 1-acetoxysafrole-2',3'-oxide > OHS-O > safrole epoxide (safrole-2',3'-oxide) ≥ 1'-oxosafrole-2',3'-oxide, the major reaction in all cases generally taking place with guanosine.

Metabolism of the related flavouring estragole (4-allyl-1-methoxybenzene), which occurs in the herbs tarragon, basil and fennel, was found to bear a close resemblance to that of safrole (Solheim & Scheline, *Xenobiotica* 1973, **3**, 493). When 100 mg/kg was given by the oral or ip route to rats, the major metabolic reactions were found to be *O*-demethylation to 4-allyl-1-hydroxybenzene and oxidation of the allylic side chain to 2-hydroxy-3-(4-methoxyphenyl)propionic acid and 4-methoxybenzoic acid (anisic acid), which was largely excreted as 4-methoxyhippuric acid. The first metabolite accounted for 39% of an oral dose and 46% of an ip dose in the 48-hour hydrolysed urine, and a further 17% of an oral dose and 31% of an ip dose was represented by the other three compounds. Small amounts of 3-hydroxy-3-(4-meth-

oxyphenyl)propionic acid were also produced, probably as a result of hydration of 4-methoxycinnamic acid. The 2-hydroxy-3-(4-methoxyphenyl)propionic acid (an α-hydroxy acid) arose via epoxidation of the allylic group to form 3-(4-methoxyphenyl)propylene-1,2-oxide, which was detected in small quantities in the bile, and subsequent hydration to a diol, 3-(4-methosyphenyl)1-,2-propanediol, which was found in both bile and urine. Administration of the epoxide resulted, as expected, in the excretion of both the diol and the α-hydroxy acid. Another point of similarity with safrole was the formation of the 1′-hydroxy derivative, 1-methoxy-4-(1′-hydrosyallyl)-benzene (1′-hydroxyestragole; OHE) which was found in both urine and bile and appeared to account for at least 5–10% of the dose.

Subsequent work (Drinkwater *et al. J. natn. Cancer Inst.* 1976, **57**, 1323) showed that when a single ip injection of estragole (1·85 mmol/kg) was given to pre-weaning or adult male mice, about 23% of the dose was excreted in the urine within 24 hours as a conjugate of OHE, together with small amounts (below 1%) of free OHE. From the same dose level of safrole only 12% was excreted as OHS by pre-weaning males, but as 46% was excreted by adult males. The latter value was higher than that reported earlier (*Cited in F.C.T.* 1974, **12**, 417), a discrepancy attributed to improved methodology. When male mice were given a total dose of 4·43 or 5·2 μmol estragole or OHE, or 4·43 μmol OHS, in four sc injections on days 1–22 after birth, only 77% of those on the lower dose and 25% of those on the higher dose of OHE survived to weaning on day 22 (Drinkwater *et al. loc. cit.*). All but the last group were maintained for a further 15 months, and the incidence of tumours was then calculated as a percentage based on the number of survivors at 12 months. Hepatocellular carcinomas were found in 23% of mice given the lower dose and in 39% given the higher dose of estragole, in 70% of those given the lower dose of OHE and in 59% of those given OHS. A similar trend was evident in the incidence of multiple liver tumours, which were found in 5, 28, 64 and 39% respectively of the mice in the four groups. In control mice injected only with the vehicle (trioctanoin) the incidence of liver tumours was only 12%, and none of these were multiple.

In vitro 1′-acetoxyestragole (AcOE) and AcOS showed a similar electrophilic reactivity towards nucleosides, both reacting non-enzymatically to the extent of 20% with guanosine and 4–5% with inosine (Drinkwater *et al. loc. cit.*). The reactivity of another allylarene, 1′-acetoxy-4-allyl-1-methoxynaphthalene (AcO-AMN) in this respect was considerably greater, but 1′-acetoxyallylbenzene (AcO-AB) showed little or no electrophilic reactivity, AcOS, AcOE and AcO-AMN demonstrated dose-related mutagenic activities of similar magnitude in *Salmonella typhimurium* strain TA100, whereas AcO-AB was only marginally mutagenic and OHS and OHE were non-mutagenic, even when incubated with liver microsomes stimulated by pretreatment with polychlorinated biphenyls (Drinkwater *et al. loc. cit.*). Provision of an NADPH-generating system also failed to elicit any mutagenic activity from either OHS or OHE, and it seemed probable that a slow rate of conversion to electrophilic metabolites *in vitro*, as demonstrated by Wislocki *et al.* (*loc. cit.*) for OHS, may have been responsible for this lack of effect.

The significance of these findings to man ingesting small quantities of either safrole or estragole has yet to be determined. In a limited metabolic study in which single oral doses of 0·163 or 1·655 mg safrole were given to eight human volunteers, the chief urinary metabolite was a conjugate of 1,2-dihydroxy-4-allylbenzene, with small amounts of eugenol or its isomer 1-methoxy-2-hydroxy-4-allylbenzene, and no OHS could be detected (Benedetti *et al. Toxicology* 1977, **7**, 69). Furthermore, when rats were given oral doses in the range of 0·6–750 mg/kg a marked decrease in the rate of elimination occurred with increasing dose, and plasma and tissue concentrations of safrole and its metabolites remained elevated for far longer at high dose levels, suggesting impairment of the degradation/excretion pathways. The proportion of the radioactive peak corresponding to 3′-hydroxysafrole increased markedly at high doses, and, as this compound is thought to arise from isomerization of OHS during enzyme hydrolysis, it suggested an increased formation of OHS with increasing dose.

These findings correlate interestingly with the results of a rat feeding test in which dietary levels in the range 0–5000 ppm safrole were administered to rats for 2 years (Long *et al. Archs Path.* 1963, **75**, 595). Liver damage was very slight at 100 ppm, slight at 500 ppm, slight to moderate at 1000 ppm and moderate to severe at 5000 ppm. Only at the two highest levels did significant numbers of liver tumours develop, and only at 5000 ppm were a large proportion malignant. Similar results were recorded in another 2-year rat study with safrole at dietary levels of 1000–10,000 ppm (Hagan *et al. Toxic. appl. Pharmac.* 1965, **7**, 18). It appears possible, therefore, that carcinogenic metabolites are produced from safrole only at high doses, at which a primary metabolic pathway may become saturated, or that at low doses any carcinogenic metabolites are produced too slowly and eliminated too rapidly to exert any biological effects. These possibilities are obviously worthy of further investigation in more detailed studies of dose-related metabolism and toxicity. Far less attention has been devoted to estragole, but similar conjectures may be made on the basis of the data available so far.

[C. Rostron—BIBRA]

THE MICRONUCLEUS TEST

Most of the currently used short-term tests for carcinogenicity depend basically on observations linked with genetic change and are thus useful also for the screening of compounds for potential mutagenic activity. Conversely, screening tests for potential mutagenicity may also find a place in the battery of tests

useful for the screening of carcinogens. One of the more promising tests in this category is the micronucleus test, which was originally devised by Matter & Schmid (*Mutation Res.* 1971, **12**, 417) as a test for detecting cytogenetic damage.

The micronucleus, as its name implies, is a nucleus-like body, generally smaller than the cell nucleus. It originates from the nucleus of a mammalian cell when the chromosomes or the cytoplasmic spindles on which the chromosomes are oriented are damaged in the course of cell division. Cells from rapidly dividing tissues, such as bone-marrow, give rise to increased numbers of micronuclei after treatment with appropriate chemical agents, but the same agents rarely induce a similar increase in other organs with a much slower rate of cell turnover.

Micronuclei are difficult to detect in nucleated cells. Not only do they vary considerably in size, so that they may be mistaken for the cell nucleus, but in many instances they may lie under or over the true nucleus and thus excape attention. In fact, the micronucleus test is a practicable proposition for screening mutagens only because micronuclei persist in young erythrocytes, which are normally anucleate. In the development of an erythrocyte, the precursor cell (erythroblast) expels its nucleus a few hours after completion of the last mitosis, but for unexplained reasons retains any micronuclei, which become very obtrusive structures. Micronuclei are present in a small proportion of the erythrocytes in untreated animals and man. These micronucleated cells occur mainly in the bone marrow but some are found in the circulation, and micronuclei have been known to haematologists for many years. In haematological terminology they are called Howell-Jolly bodies.

Because micronuclei reflect some damage to the genome, it was thought that they might have a place in the identification of mutagenic agents, and a technique was developed for their detection and quantitation in the bone marrow of various experimental animals (Von Ledebur & Schmid, *ibid* 1973, **19**, 109). For routine studies, mice, rats or hamsters are generally used, since larger animals are less convenient, more expensive and less easy to obtain. Adolescent animals (aged 7–12 weeks) are recommended, their marrow being free of the excess of fat found in older animals and at the same time lacking the high proliferation rate in the constituent cells found in younger animals. Experience has shown that both of these factors interfere with the quality of the marrow smears. Bone-marrow smears are more suitable than blood smears for these studies because the polychromatic (young) erythrocytes are more numerous in the marrow.

In practice, the test is carried out by administering the test compound to the adolescent animals (commonly mice of an outbred strain) either orally or, more often, parenterally, 30 hours before the animals are killed for preparation of bone-marrow smears. Originally only this single treatment was recommended, but with greater experience it has been found that better results can be obtained by administering a second dose 6 hours before the animals are killed. Bone-marrow smears are prepared using a conventional haematological stain (May-Grünwald/Giemsa), and 2000 or more polychromatic erythrocytes, which are recognized by their bluish staining characteristics, are screened for the presence of micronuclei.

Positive results with this technique were given by the alkylating agents Trenimon (2,3,5-tri(ethylenimino)-*p*-benzoquinone), cyclophosphamide, triethylenemelanine, methyl methanesulphonate, ethyl methanesulphonate and triethylenethiophosphoramide (Thio-TEPA), by the cytostatic antibiotics mitomycin C and adriamycin, by the spindle poisons colchicine, colcemid and vincristine, and by the antimetabolites 6-mercaptopurine, methotrexate, 5-fluorouracil and 1-β-D-arabinofuranosylcytosine (Maier & Schmid, *ibid* 1976, **40**, 325; Matter & Schmid, *loc. cit.*), while negative results were obtained with bleomycin, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and caffeine (Matter & Schmid, *loc. cit.*; Schmid *et al. Humangenetik* 1971, **11**, 103).

A dose-response curve was obtained with both cyclophosphamide and Thio-TEPA, over the ip dose ranges 1–500 and 0·1–100 mg/kg, respectively (Maier & Schmid, *loc. cit.*). No significant increase above the background level of four micronucleated cells/1000 polychromatic erythrocytes was obtained with two doses of 1 mg cyclophosphamide/kg but doses of 100 mg/kg produced a level of 55/1000 and five times this dose produced 83/1000. The figures for Thio-TEPA were 20/1000 with a dose of 1 mg/kg and 47/1000 with either 10 or 100 mg/kg. According to Schmid (in *Chemical Mutagens*, vol. 4. edited by A. Hollaender; p. 31; Plenum Publishing Corp., 1976), the curve obtained with cyclophosphamide is characteristic of compounds requiring metabolic activation.

The spindle poison vincristine produced a totally different type of curve. Doses of 0·01, 0·05, 0·1 and 0·2 mg/kg produced 0, 20, 39 and 65 micronucleated cells/1000 polychromatic erythrocytes. On the other hand, higher doses, 0·5, 1·0 and 10·0 mg/kg, produced only 37, 14 and 10/1000, respectively (Maier & Schmid, *loc. cit.*). The reason for this seemingly anomalous result is not altogether clear, but it is thought that at the lower doses, impairment of the spindle is only partial and causes loss of single chromosomes with production of micronuclei, while at the higher doses, the spindle apparatus is blocked completely and the nuclei enlarge and are expelled *in toto* without leaving micronuclei behind (Schmid, *loc. cit.*).

In assessing this test, the latter author pinpoints the major advantages as the simplicity of the technique, which obviates the need for special cytogenetic training such as is required for recognizing abnormal chromosomes, the low and consistent background level of micronucleated erythrocytes, and the relatively short duration of the test. The main disadvantages are its inability to detect weak mutagens, such as those capable of producing only specific point mutations, and to give a positive effect with some substances (such as bleomycin and MNNG) known to cause chromosomal changes *in vitro*. There is also the problem, common to many tests carried out in mammalian systems, that with substances requiring metabolic activation, an insufficient quantity of the reactive metabolite may reach the bone marrow.

There is little to dispute in this view of the advantages and limitations of the micronucleus test, but a final assessment must await the completion of more extensive validation studies. The negative results

obtained with MNNG, a potent proximate mutagen and carcinogen, indicate that there may be certain reactive groups of compounds to which the test is insensitive, and it is essential to extend the range of materials tested in order to identify more closely the areas in which the micronucleus test is unlikely to be of value. Only then will there be any possibility of combining this test with other appropriate short-term methods to provide a battery of tests that could reasonably be expected to cover the whole front and provide an effective primary screen for detecting mutagens and carcinogens.

[P. Grasso—BIBRA]

Fd Cosmet. Toxicol. Vol. 15, pp. 649-656. Pergamon Press 1977. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

3224. Brown FK mutagenic in TA1538 only

Venitt, S. & Bushell, C. T. (1976). Mutagenicity of the food colour Brown FK and constituents in *Salmonella typhimurium*. *Mutation Res.* **40**, 309.

The characteristic brown colour of most kippered herrings produced in the UK is due to the presence of a synthetic dye, Brown FK, which is essentially a mixture of six *p*-sulphophenylazo derivatives of *m*-toluene- and *m*-phenylenediamine. The structure of these consitiuents has led to speculation about possible carcinogenicity, but such suspicions have not been confirmed by experiments carried out to date. To add to the debate, the authors cited above undertook to test the colouring for mutagenic activity in bacteria, using the now familiar Ames test (*Cited in F.C.T.* 1976, **14**, 353).

Three strains of *Salmonella typhimurium* were used, namely TA1535 for detecting base-pair substitutions and both TA1537 and TA1538 for frameshift mutagens. Samples of Brown FK from three manufacturers were mutagenic in TA1538 when activated by a supernatant fraction of rat-liver homogenate, but not in the other two strains. The effect in TA1538 was linearly dose-dependent in the range 0–3 mg/plate, with activities between 22 and 50 times the spontaneous mutation frequency. One sample of Brown FK showed some mutagenicity in the absence of metabolic activation, producing a 16-fold increase in mutation rate at a dose of 4 mg/plate.

Two of the major constituents of Brown FK, 2,4-diamino-5-(*p*-sulphophenylazo)toluene and 1,3-diamino-4-(*p*-sulphophenylazo)benzene were mutagenic in TA1538. Mutagenicity was again linearly related to dose, in the range 0–1 μmol/plate, and the activity was dependent on metabolic activation. These compounds together account for some 36% of the colouring and were the two constituents associated earlier with its myotoxicity (*Fd Cosmet. Toxicol.* 1970, **8**, 539). The other di- and tri-substituted diamines that comprise Brown FK were inactive, as was sulphanilic acid, the major excretion product in experimental animals.

[The results of long-term carcinogenicity studies on Brown FK carried out in rodents were examined by the EEC Scientific Committee for Food (SCF) in 1975. The data gave no indication that the colouring was carcinogenic, but the Committee requested the results of a further long-term study in another strain of rat, with accompanying records of the nitrite level in the animal diets. In the same report, the SCF supported the verdict of a WHO Scientific Group that *in vitro* mutagenicity tests alone cannot yield definitive results applicable to man.]

AGRICULTURAL CHEMICALS

3225. Long-term effects of ethylene thiourea

Gak, J.-C., Graillot, C. et Truhaut, R. (1976). Différence de sensibilité du hamster et du rat vis-à-vis des effets de l'administration à long terme de l'éthylène thiourée. *Eur. J. Toxicol.* **9**, 303.

Ethylene thiourea (ETU) is used as a rubber accelerator, and is also formed as a degradation product of the ethylenebisdithiocarbamate fungicides. In mice, a dose level of 215 mg/kg given by stomach tube on days 7–28 after birth, followed by 646 ppm in the diet for 18 months, produced an increased incidence of hepatomas and lymphomas (Innes *et al. J. natn. Cancer Inst.* 1969, **42**, 1101), while in rats fed dietary levels of 175 or 350 ppm for 18 months there was a dose-related increase in thyroid lesions, including carcinomas (*Cited in F.C.T.* 1973, **11**, 702). When dietary levels in the range 5–500 ppm were fed to rats for 2 yr, slight thyroid hyperplasia was seen at 5 and 25 ppm, but only at 125 ppm and above was there evidence of tumour formation (*Fd Cosmet. Toxicol.* 1975 **13**, 493). ETU has also been implicated as a teratogen in rats and rabbits (*Cited in F.C.T.* 1977, **15**, 157; *ibid* 1977, **15**, 361). However, in a limited epidemiological survey among industrial users of ETU, no evidence of teratogenicity or thyroid cancer was found (*ibid* 1977, **15**, 363). A further study of the long-term, low-level effects of ETU in animals has now been reported.

When dietary levels of 5, 17, 60 or 200 ppm ETU were administered to rats for 2 yr and to hamsters for 20 months, growth was significantly retarded in the rats by 17 ppm and in the hamsters by 60 ppm, and there was an accompanying reduction in food intake. Serum levels of the liver enzyme glutamic–pyruvic transaminase (GPT) showed a transient increase in all treated rats, particularly in the males, but only in the males on the two higher levels was there an initial increase in the alkaline-phosphatase (AP) activity of the serum. In the hamster, serum GPT was unaffected by ETU ingestion, while serum AP in all ETU-treated groups first increased and then decreased relative to levels in controls. In both species there was a marked dose-related increase in serum cholesterol, which even with only 5 ppm in the diet has risen by about 30% after 3 months. After 20 months, liver analyses had revealed an increase in

GPT and AP activities and a decrease in glucose 6-phosphate dehydrogenase following all levels of treatment in the hamster, but in the rat the latter enzyme was unchanged and AP and GPT were increased significantly only in the males on the higher dietary levels.

Thyroid weight was increased significantly by 60 ppm ETU or more in the rat, but only by 200 ppm in the hamster (although the difficulty of removing the thyroid in the latter species made this result questionable). On histological examination, the hamsters showed extensive dose-related degeneration of the bile ducts but no thyroid lesions, while in the rats the liver histology was unremarkable but numerous haemorrhagic cysts were found in the thyroid in animals fed 60 ppm ETU or more. The incidence of carcinomas, predominantly in the thyroid but also to a lesser extent affecting the Leydig cells in the testes, was significantly increased in male rats given 60 ppm and in female rats given 200 ppm, and females given 17 or 60 ppm also showed some increase, although this was not statistically significant. However, in hamsters there was no evidence of carcinogenicity at any dose level. A further study now in progress is aimed at establishing a no-effect level and determining whether the observed toxic effects can all be ascribed to a primary action on the thyroid.

3226. Inhaling pentachlorophenol

Hoben, H. J., Ching, S. A. & Casarett, L. J. (1976). A study of inhalation of pentachlorophenol by rats. III. Inhalation toxicity study. *Bull. env. contam. & Toxicol. (U.S.)* **15**, 463.

Hoben, H. J., Ching, S. A. & Casarett, L. J. (1976). A study of inhalation of pentachlorophenol by rats. IV. Distribution and excretion of inhaled pentachlorophenol. *Bull. env. contam. & Toxicol. (U.S.)* **15**, 466.

Pentachlorophenol (PCP) and its sodium salt (Na PCP) are widely used as wood preservatives and in other fungicidal and pesticidal products. Aplastic anaemia, prostration, paralysis and sensory loss have resulted from over-exposure in industrial workers, in whom skin absorption is a major route of entry (*Cited in F.C.T.* 1965, **3**, 846). Toxic effects have also resulted in infants from percutaneous absorption of PCP used to launder nappies (*ibid* 1971, **9**, 601). Very recently, sickness and death in Michigan cattle have been tentatively associated with residues of PCP and its chlorinated dioxin contaminants found in the blood, liver and fat of the affected animals (*Fd. chem. News.* 1977, **19**, (1) 36). Rats given a single oral dose of PCP excreted a large proportion of the dose in the urine between 16 and 20 hr after treatment, and after 10 days 68% had been eliminated by this route and a further 9–13% in the faeces (*Cited in F.C.T.* 1973, **11**, 1152). An even greater proportion (70%) of an ip dose was excreted in the urine in the first 24 hr, as free or conjugated PCP and tetrachlorohydroquinone (TCHQ), the metabolites that were detected also after oral administration (*ibid* 1975, **13**, 585).

In the first paper cited above, Hoben *et al.* reported LD_{50} values for Na PCP in the rat of 34 mg/kg ip and 11·7 mg/kg by inhalation, the latter being determined by exposure to an aerosol for 28–44 min. These values were considerably below the previously reported LD_{50} values for Na PCP in the rat of 211 mg/kg by the oral and 66 mg/kg by the sc route (Deichmann *et al. J. Pharmac. exp. Ther.* 1942, **76**, 104). The reason for the high toxicity of the inhaled material, and its potential for accumulation, were explored in the second study cited above.

Rats were given a single 20-min exposure to an aerosol of Na PCP, the concentration in the inhaled air being such as to give each animal a calculated dose of 5·7 mg/kg, or were exposed for 20 min daily on up to 5 successive days, the calculated inhaled doses being 5·9 mg/kg/day for two, three or four exposures and 1·04 mg/kg/day for five. Levels of PCP in the lung, plasma and liver were determined at intervals of 0–72 hr after exposure and the urine also was analysed over this period. In tissue and fluid analyses carried out 24 hr after the single exposure, the unchanged PCP identified accounted for about 73% of the dose, approximately 55% of the dose being in the urine, 9% in the liver, 8% in the plasma and 0·8% in the lung; only traces of TCHQ were detectable. In contrast, after ip administration only 30–40% of the dose was accounted for as unchanged PCP, and about the same amount again was present in the liver and urine as TCHQ. After inhalation the clearance rates from all tissues and fluids analysed were essentially the same, indicating the absence of storage or preferential binding at these sites. Clearance appeared to follow first-order kinetics, with a half-life of about 24 hr. Despite this, levels in lung, plasma and liver did not increase on repeated exposure, and in fact showed some tendency to decrease when measured 24 hr after each exposure. Meanwhile the percentage excreted in the urine showed an apparent increase of only about 20% although the wide standard error made the significance of this finding uncertain.

The lack of either an extended elimination period or a significantly increased elimination rate after repeated inhalation suggested that PCP was not being stored in a non-analysed site, such as the body fat, and it appeared likely that instead it was undergoing accelerated biotransformation as a result of enzyme induction. Further studies would, however, be necessary to confirm this hypothesis.

THE CHEMICAL ENVIRONMENT

3227. Lead from kettles

Ng, R. & Martin, D. J. (1977). Lead poisoning from lead-soldered electric kettles. *Can. med. Ass. J.* **116**, 508.

It was discovered in 1974 that some Canadian electric kettles released high levels of lead, apparently from the solder between the heating element and the base of the kettle. The FDA then investigated imported kettles and recalled some kettles releasing

more than the Canadian guideline of 0·05 ppm lead into the water (*Fd. chem. News.* 1974, **16**, (25) 28). Two cases of lead poisoning in infants fed on formulations prepared with water boiled in lead-soldered kettles are described in the paper cited above.

The first case was a 3-month-old girl, who suffered from repeated convulsions lasting up to 30 min. Her symptoms included projectile vomiting, lethargy and irritability. She was well-developed and well-nourished and all systems appeared normal. However X-rays of the skull and chest showed dense metaphyseal bands, splitting of the sutures and increased density of the sutural margins. X-rays of the wrists, knees and abdomen also showed lines of increased density at the metaphyses compatible with heavy-metal poisoning. The blood-lead level was 145 μg/100 ml, and tests for urinary coproporphyrins were strongly positive. Chelation therapy with dimercaprol and ethylenediaminetetraacetic acid for 5 days caused excretion of large amounts of lead and δ-aminolaevulinic acid in the urine. The blood-lead level was 60 μg/100 ml when the child was discharged and 18 months later she was completely normal. The public health authority discovered that water used to prepare the infant's feed was boiled in a lead-soldered electric kettle and contained 120 μg lead/100 ml; thus she had been ingesting about 600 μg lead/day, which is 12 times the maximum level generally considered to be safe.

The condition of the second case, a 9-month-old boy, was found by chance when he was admitted for treatment of severe seborrhoeic dermatitis. X-rays of his skull and long bones showed dense metaphyseal bands and heavy-metal poisoning was suspected. His blood-lead level was 35 μg/100 ml, the level of free erythrocyte protoporphyrin was 750 μg/100 ml (ten times the normal value) and the δ-aminolaevulinic acid level was 150 μmol/100 ml of erythrocytes (0·25 times the normal value). EDTA-mobilization testing revealed 372 μg lead in the urine collected for 24 hr after administration of 200 mg EDTA, confirming lead poisoning. Treatment with D-penicillamine for 2 days had to be stopped because of its probable involvement in the development of a severe rash. However, it was found that the mother had been preparing the infant's feed with water boiled in a kettle which was a type amongst those listed by the government as releasing more than 5 μg lead/100 ml water. In fact this kettle was releasing 75 μg lead/100 ml. With the elimination of this source of lead, about 375 μg/day, the patient's blood-level soon decreased to normal. A year later the 'lead-lines' had nearly gone from his bones, the level of free erythrocyte protoporphyrin was declining and he remained asymptomatic.

Potential sources of lead must be detected and eliminated from an infant's restricted diet. Both the boiling of liquids in lead-soldered kettles and the use of lead solder in cans used for evaporated milk increase the chances of lead poisoning in the very young. Such poisoning is difficult to detect and may only be discovered by chance, as in the second case described above. These cases illustrate the importance of the dense metaphyseal bands observed in bone X-rays as a clue to the condition.

3228. Carcinogenicity of metals

Stoner, G. D., Shimkin, M. B., Troxell, M. C., Thompson, T. L. & Terry, L. S. (1976). Test for carcinogenicity of metallic compounds by the pulmonary tumor response in strain A mice. *Cancer Res.* **36**, 1744.

Tumour induction by metal compounds was thoroughly reviewed by Sunderman (*Fd Cosmet. Toxicol.* 1971, **9**, 105) who described reports of tumours produced in experimental animals by metallic compounds, including compounds of cadmium, chromium, cobalt, iron (in the form of carbohydrate complexes), lead, nickel and zinc. Nickel is probably the most widely documented of these, having been additionally implicated in nasal and lung cancer in man (*Cited in F.C.T.* 1969, **7**, 693; *ibid* 1972, **10**, 113; *ibid* 1977, **15**, 362).

In the work cited above, groups of 20 mice (ten male and ten female) were given up to 24 thrice-weekly ip injections of cadmium acetate, calcium acetate, chromium sulphate, cobalt acetate, cupric acetate, iron 2,4-pentanedione, lead subacetate, manganous sulphate, molybdenum trioxide, nickelous acetate, stannous chloride, vanadium 2,4-pentanedione and zinc acetate. Fewer injections were given of the more toxic chemicals. The three dose levels used were the maximum tolerated dose (estimated from preliminary toxicity tests) and 1:2 and 1:5 dilutions of the maximum tolerated dose. Thus doses ranged from 7 mg cadmium acetate/kg to 4750 mg molybdenum trioxide/kg. The highest doses of cadmium acetate and iron 2,4-pentanedione used killed all the treated animals owing to delayed toxicity. All the surviving mice were killed 30 wk after the first injection and their lungs were removed and fixed. The nodules on the lungs were then counted and some were examined histopathologically. The liver, intestines, thymus, kidney, spleen and salivary and endocrine glands were also examined for abnormal lesions.

Of the 13 compounds tested, four (lead subacetate, manganous sulphate, molybdenum trioxide and nickelous acetate) produced a significant increase in the number of lung tumours compared with controls and there was a well-defined dose-response relationship between the intermediate and high doses, although the effects of the low and intermediate doses were similar. The highest dose of cupric acetate produced an average of two lung tumours per mouse, the highest number recorded, but this was based on only five surviving animals and thus was not statistically significant. A total of seven adenocarcinomas occurred, five in mice receiving nickelous acetate and two in groups receiving vanadium. On a molar basis, lead subacetate was the most active compound, 0·185 nmol/kg producing 1·47 tumours/mouse; nickelous sulphate had the second highest activity, followed by manganous sulphate and molybdenum trioxide. The only tumours observed at other sites were four thymomas in mice treated with zinc acetate and one salivary-gland tumour in a mouse treated with chromium acetate, but the incidence of these tumours was not statistically significant.

The results with the nickel and lead salts support other findings of tumour induction by parenteral administration of compounds of these two metals to

mice, in spite of the reported lack of response of Swiss mice to life-time administration of nickel or lead salts in the drinking-water (*Cited in F.C.T.* 1965, **3**, 536). The inactivity of cobalt acetate, iron 2,4-pentanedione, zinc acetate and cadmium acetate may have been related to their toxicity at relatively low dose levels.

3229. Benzene leukaemia detailed

Vigliani, E. C. & Forni, A. (1976) Benzene and leukemia. *Envir. Res.* **11**, 122.

The insidious dangers of excessive exposure to benzene have been established for a number of years, but unfortunately the time scale of the leukaemia process is such that data on the solvent's carcinogenicity is still being generated. The review cited above assesses in some detail 150 cases of benzene-induced leukaemia reported up to the beginning of 1975.

Leukaemia is often observed in individuals who have worked in factories where there have been outbreaks of benzene poisoning. In the shoe factories of Lombardy, 33 of 83 workers who suffered acute benzene poisoning have died, 14 of the deaths being due to aplastic anaemia and 18 to acute leukaemia, together with a single case of erythroleukaemia. In general benzene leukaemias are usually of the acute form, either haemocytoblastic or myeloblastic in type, and very often the disease develops in subjects with hyporegenerative anaemia or pancytopenia. Aplastic anaemia generally occurs when the subject is still exposed to the solvent, while leukaemia often develops only after termination of exposure.

Of 11 cases of benzene leukaemia seen at the Milan Institute of Occupational Health since the war, seven were classified as acute haemocytoblastic or myeloblastic leukaemias, one as acute erythraemia and three as erythroleukaemia. Although earlier reviewers attributed chronic myeloid and chronic lymphocytic leukaemias to benzene, the present authors consider these to be unusual reactions to benzene exposure. For example, 16 deaths have occurred in a sample of 142 Italian workers who suffered chronic benzene poisoning, and of these, three were due to aplastic anaemia and 13 to leukaemia, in each case of the acute type. Similarly the four cases of leukaemia observed in 35 Turkish shoe workers with hyporegenerative anaemia were all acute in nature (three myeloblastic and one monocutic). It has been suggested that in workers heavily exposed to benzene the chance of contracting acute leukaemia is at least 20 times greater than in the general population.

The mechanism of benzene's leukaemogenic action is also discussed in the review. Benzene induces chromosomal changes in both lymphocytes and bone-marrow cells. It has been suggested that the latter may result in cells with an abnormal chromosome complement and thus give rise to leukaemic clone. However, even if benzene does transform cells directly, it is still uncertain whether it acts by a mutagenic mechanism or has an effect on gene expression. A further possibility is that it may induce an impairment of the immune response, and so reduce the efficiency of elimination of abnormal cell clones.

3230. Methylene chloride and ethanol synergism

Balmer, M. F., Smith, F. A., Leach, L. J. & Yuile, C. L. (1976). Effects in the liver of methylene chloride inhaled alone and with ethyl alcohol. *Am. ind. Hyg. Ass. J.* **37**, 345.

Although there are as yet no adequate long-term data on methylene chloride (CH_2Cl_2), it is thought to be one of the least hepatotoxic of the chlorinated solvents. At least four long-term toxicity studies are in progress, including two inhalation studies (*Tox Tips* 1976, **1** (6), 7 & (7), 21), and the results of these should greatly facilitate the definition of safe industrial limits. However, factory atmospheres often contain several potentially hazardous materials and it is thus important to be aware of the possible toxicological consequences of common solvent combinations. The study cited above investigated the biological activity of CH_2Cl_2 in the presence of ethanol.

Male guinea-pigs were exposed for 6 hr daily to atmospheres of CH_2Cl_2, ethanol or ethanol–CH_2Cl_2 mixtures for 1 or 5 days. In the acute experiment, four of ten guinea-pigs died when exposed to 11,100 ppm CH_2Cl_2, but simultaneous exposure to 11,100 ppm CH_2Cl_2 and 25,100 ppm ethanol produced no deaths. The antagonism between the two chemicals was also reflected in the liver-fat and blood-carboxyhaemoglobin (COHb) levels, both of which were lower in the animals exposed to the mixture than in those exposed to CH_2Cl_2 alone.

In contrast, potentiation between ethanol and CH_2Cl_2 was observed in the subacute experiment. No deaths occurred in five animals exposed on five consecutive days to 500 ppm CH_2Cl_2, whereas one animal died (on day 3) when exposed to a combination of 500 ppm CH_2Cl_2 and 25,000 ppm ethanol. Animals subjected to ethanol (25,000 ppm), to CH_2Cl_2 (500 ppm) or to both solvents simultaneously lost 50, 10 and 72 g, respectively, in body weight over the 5 days, compared with a weight gain of 18 g in the controls. In all three treated groups the hepatic triglyceride levels were raised in comparison with non-exposed controls. Histologically, moderate fatty changes were found in one animal exposed to 500 ppm CH_2Cl_2 and in three exposed to 25,000 ppm ethanol, whereas a diffuse fatty change was noted in four of the guinea-pigs exposed repeatedly to a combination of the solvents.

While a single exposure to CH_2Cl_2 at 560 ppm raised COHb levels above those of the controls, these levels were not significantly elevated in animals exposed on 5 days to either 500 ppm CH_2Cl_2 or to the CH_2Cl_2–ethanol mixture. Tolerance to CH_2Cl_2 has not been reported in earlier studies in the rat or man.

In the subacute experiments, the observed pattern of liver pathology suggested that ethanol was the causative agent and the potentiation was possibly due to the irritant effect of CH_2Cl_2 on the lungs and a consequent increase in ethanol absorption. It is suggested that the antagonism noted in the acute experiment may have been a consequence of competition between the two solvents for a common enzyme system, resulting in the metabolism of only "ineffective"

amounts of each to the proximate toxin (perhaps a free radical).

[Previous work in the rat and observations in man have indicated that regular exposure to methylene chloride increases blood levels of carboxy-haemoglobin. The limited evidence derived from the present study indicates that similar blood changes do not occur in the guinea-pig, suggesting that this species is not a good model for investigating possible human reactions to this solvent.]

NATURAL PRODUCTS

3231. Congeners and alcohol metabolism

Auty, R. M. & Branch, R. A. (1976). The elimination of ethyl, *n*-propyl, *n*-butyl and iso-amyl alcohols by the isolated perfused rat liver. *J. Pharmac. exp. Ther.* **197**, 669.

Although the congeners present in alcoholic beverages may enhance the neuropharmocological effects of alcohol, several studies have revealed no evidence that they also enhance its hepatotoxic effects (*Cited in F.C.T.* 1975, **13**, 591 & 676). Among the most common congeners are *n*-propanol, *n*-butanol and isoamyl alcohol, all of which, like ethanol, are substrates for the liver enzyme, alcohol dehydrogenase (ADH). Large doses of *n*-propanol or of fusel oil, which contains these four and other alcohols, inhibited [1-^{14}C]ethanol oxidation to $^{14}CO_2$ in the rat, although with low doses of fusel oil the oxidation process was accelerated (Aebi & von Wartburg, *Bull. schweiz. Akad. med. Wiss.* 1960, **16**, 25; von Wartburg *et al. Biochemistry, N.Y.* 1964, **3**, 1775). The possibility that the three higher homologues might affect ethanol elimination has therefore been investigated.

When perfused through the isolated rat liver, ethanol, *n*-propanol and *n*-butanol were eliminated at a rate that followed zero-order kinetics at high concentrations and first-order (exponential) kinetics at low concentrations. The critical concentration at which the change in kinetics occurred decreased with increasing carbon-chain length, from 5 mmol for ethanol to 1·3 mmol for *n*-propanol and 0·8 mmol for *n*-butanol. Isoamyl alcohol followed first-order kinetics below 1 mmol, but above this concentration data were insufficient to permit any firm conclusions. The half-life of the first-order phase ranged from approximately 14 min for both ethanol and *n*-propanol to 3 min for the lowest concentration of isoamyl alcohol studied (0·4 mmol), while the rate of the zero-order phase declined from 10·5 mmol/min for ethanol to 3·9 mmol/min for *n*-propanol and 3·3 mmol/min for *n*-butanol. The apparent Michaelis constants for the alcohols also decreased as the length of the carbon chain increased, and were similar to those previously reported from *in vitro* experiments with ADH.

When ethanol (10 mmol) was perfused simultaneously with *n*-propanol, *n*-butanol or isoamyl alcohol at concentrations of 2·3, 1·9 and 1·2 mmol, respectively, there was a decrease in the rate of both phases of ethanol elimination, and the elimination rate of the three higher alcohols was also decreased. The apparent Michaelis constant of all four alcohols was increased under these circumstances, but the apparent maximal velocity of their elimination remained unchanged. Findings suggested that the higher alcohols were competing with ethanol for the rate-limiting process of oxidation by ADH. However, their concentrations in the perfusate, relative to that of ethanol, were almost two orders of magnitude greater than would be found in a man who had been drinking a typical alcoholic beverage, and the significance of the results for the moderate drinker is thus uncertain.

3232. Wool dust and the lungs

Žuškin, E., Valic, F. & Bouhuys, A. (1976). Effect of wool dust on respiratory function. *Am. Rev. resp. Dis.* 114, 705.

The inhalation of cotton dust by textile workers is known to cause decreases in the ventilatory capacity of the lungs as well as symptoms of chest tightness and dyspnoea. In recent years it has been shown that byssinosis is relatively common among cotton-textile workers (*Cited in F.C.T.* 1972, **10**, 861) and this has led to an increased interest in this syndrome and in the nature of the airway responses that accompany the inhalation of textile dusts.

The above-cited study examines the acute and chronic changes in lung function in a group of workers exposed to wool dust in two textile mills in Yugoslavia. In addition the investigators examined a control group of residents living in the neighbourhood of the mills and a small group of healthy subjects exposed experimentally to extracts of wool and cotton dust. Ventilatory function was measured by recording maximal expiratory flow (MEF)/volume curves and forced expiratory volume in 1 sec (FEV_1) before and after the work shift on the first working day of the week. From the MEF/volume curves, the flow rates at 50% of the control vital capacity were read. The experimental subjects for the cotton- and wool-dust comparative studies had been shown to be 'reactors' in previous inhalation experiments with extracts of cotton or hemp dust. Each was exposed for 10 min to a dust extract in the form of an aerosol containing particles not exceeding 0·5 μm in diameter.

Workers exposed to wool dusts for longer than 10 yr showed more signs of chronic respiratory effect than did those exposed for less than 10 yr, although the difference was not statistically significant. Significant reductions during the work shift were observed in MEF rates at 50% of the control vital capacity and in FEV_1. The first of these tests proved the more sensitive. Individuals (smokers as well as non-smokers) exposed to wool dust for more than 10 yr had significantly lower than predicted pre-shift values for MEF rates at 50% of the control vital capacity.

The comparison of the effects of wool- and cotton-dust extracts in healthy volunteers indicated a similar acute effect of both dust extracts during the 40-min period after exposure. However, a significantly smaller reaction to the wool-dust extract in the period 60–100 min after the challenge indicated either that wool dust released less constricting agent from lung tissue or that the mechanism of the wool-dust effect was different from that of cotton dust.

Although no typical case of byssinosis was found in the wool workers, respiratory difficulties on Mondays in three subjects suggested that more sensitive individuals might develop symptoms similar to those of byssinosis.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3233. Genetic damage from *p*-aminobenzoic acid?

Hodges, N. D. M., Moss, S. H. & Davies, D. J. G. (1976). Evidence for increased genetic damage due to the presence of a sunscreen agent, *para*-aminobenzoic acid, during irradiation with near ultraviolet light. *J. Pharm. Pharmac.* **28**, 53P.

p-Aminobenzoic acid (PABA) is used as a sunscreen agent, and a 5% lotion for this purpose was introduced into the British Pharmaceutical Codex in 1973. Apart from a low incidence of allergic skin reactions (*Cited in F.C.T.* 1973, **11**, 706), no adverse effects are known to have resulted from this use. However, a report that PABA can increase ultraviolet-induced damage to bacterial DNA at wavelengths present in sunlight has now been produced.

Cultures of a strain of *Escherichia coli* defective in an enzymatic repair mechanism essential for the normal maintenance of the integrity of DNA were suspended in a salts medium containing PABA at concentrations between 0·001 and 0·10%, and irradiated with 313 nm UV from a mercury lamp, at a temperature of 0°C to prevent concurrent photoenzymatic repair. Survival curves were then obtained by plating on nutrient agar. Plots of log survivors against dose showed a small shoulder at high survival levels, followed by a linear portion, and the value of the slope (k) of the latter was used as a measure of sensitivity. Calculation of the ratios of k values in the presence and absence of PABA showed that bacterial sensitivity was increased at all PABA concentrations tested, by factors ranging from 1·2 at 0·001% to 8·42 at 0·1%. The damage induced in DNA by light of the wavelength used is chiefly pyrimidine dimerization, which undergoes selective photoenzymatic repair during subsequent illumination with visible light. However, PABA exposure reduced the reparable fraction of the induced damage, suggesting that lesions other than pyrimidine dimerization played a relatively greater role in DNA damage in the treated cultures.

[The pitfalls of using bacterial tests to predict human hazards have been discussed before in these pages (*Fd Cosmet. Toxicol.* 1977, **15**, 74). It is interesting to note in this connexion that a long-term test in mice involving skin-painting with PABA at concentrations of 1–10% revealed no evidence of carcinogenicity (Stenbäck & Shubik, *Toxic. appl. Pharmac.* 1974, **30**, 7), although unfortunately the effects of concurrent UV irradiation were not investigated.]

3234. Beauty and the beast

Garfinkel, J., Selvin, S. & Brown, S. M. (1977). Possible increased risk of lung cancer among beauticians. *J. natn. Cancer Inst.* **58**, 141.

Beauticians are exposed to a wide variety of chemical substances, many organic and several potentially toxic. Amongst this group of materials are the hair dyes, which have recently been shown to give positive results in some bacterial-mammalian tissue systems developed to detect mutagenic compounds. Although there is still much discussion on the significance of such short-term tests for assessing carcinogenic potential, the study cited above was undertaken to provide a preliminary investigation of whether beauticians may be subjected to an increased risk of cancer.

A computer file of all deaths from cancer in Alameda County, California, during 1958–1962 formed the data source. This contained all death certificates on which cancer was listed as the immediate or underlying cause of death or as a significant condition present at death. Twenty-four female beauticians were located among 3460 adult females who died from cancer, whilst only four were present in a sample of 1000 females who died from causes other than cancer. Both case and control certificates were matched for age and race. The resulting odds ratio (1·73) was slightly elevated although it was not statistically significant. A possible increase in cancer risk was found for several organ sites, but the increase in risk for lung cancer was the most striking. An analysis of pairs of death certificates, in which female controls dying of causes other than cancer were matched with female lung-cancer cases for age, race and date of death, yielded an odds ratio of 6·0. This finding suggested that the risk of lung cancer may be substantially increased among female beauticians.

Relevant variables known to be associated with lung cancer are the effects of cigarette smoking, age, sex, race, urban or rural residence, certain occupations, socio-economic class and possibly air pollution. Some of these factors were dealt with adequately by the matching procedure used, but not all; cigarette smoking, for example, was omitted from this study as it was not possible to obtain accurate smoking data for persons dying 14–18 years before. The authors admit that their findings are tentative because of the small numbers involved, the absence of control for potentially confounding variables and the fact that the identification of occupation from death certificates cannot be altogether reliable.

CANCER RESEARCH

3235. Gastric cancer and nitrosamine formation

Ruddell, W. S. J., Bone, E. S., Hill, M. J., Blendis, L. M. & Walters, C. L. (1976). Gastric-juice nitrite. A risk factor for cancer in the hypochlorhydric stomach? *Lancet* **II**, 1037.

The potency of the nitrosamines as carcinogens has encouraged research into the factors controlling the formation of these compounds within the gut. *In vitro* and *in vivo* animal studies have demonstrated that nitrosamine formation occurs in a wide range of conditions, the reaction proceeding spontaneously at acid pH but with increasing enzyme involvement at higher pH values (Klubes *et al. Fd Cosmet. Toxicol.* 1972, **10,** 757). In the study cited above, the conditions in human gastric juice were considered in relation to nitrosamine formation.

Samples of gastric juice were obtained from 69 patients undergoing routine gastro-intestinal investigation. These patients were divided into four groups, comprising 30 patients with no gastro-intestinal abnormalities, 21 with duodenal ulcers, 12 with benign gastric ulcers and six with gastric cancer. The 30 individuals with no abnormalities were further divided into a 'normal' group of 18 with a stomach pH below 2·5 and the 'hypochlorhydric normals' with a stomach pH above 5·0.

The thiocyanate ion has been shown to be a potent catalyst of the nitrosation reaction (*Fd Cosmet. Toxicol.* 1971, **9,** 639). There were no consistent differences in thiocyanate levels between the four main groups in the present study, although the stomachs of smokers had significantly higher levels than did those of non-smokers. However, there was a direct relationship between the gastric pH of each group and the nitrite concentration in the stomach. Of the individuals without gastro-intestinal problems, the normal group had a low nitrite concentration, whereas the hypochlorhydric normals had a relatively high concentration. Although the gastric-cancer patients had both the highest stomach pH, apart from the hypochlorhydric normals and the highest nitrite concentration, the latter was probably not directly connected with the cancer, since it did not differ significantly from the nitrite concentration of the hypochlorhydric normals. There were also differences between the groups in their total bacterial and nitrate-reductase concentrations, but these were thought to be secondary to pH differences, a high pH producing a correspondingly high bacterial count. It was thought that the nitrate-reducing bacteria, which are active at higher pH values, were responsible for the high nitrite concentrations seen in the more alkaline gastric juices.

As the rate of formation of nitrosamine is proportional to the square of the nitrite concentration, a high stomach pH would favour the nitrosation reaction, which would proceed by an enzymatic mechanism. The average nitrite concentration in those suffering from gastric cancer was $38{\cdot}8 \pm 14{\cdot}7$ μmol/litre and although nitrite concentrations of 60 μmol/litre in the saliva have been shown to lead to nitrosamine formation, lower concentrations have yet to be tested. Earlier studies have shown an association between hypochlorhydria and gastric cancer (Wynder *et al. Cancer, N. Y.* 1963, **16,** 1461). Ruddell *et al.* (cited above) suggest that the bacteria present in neutral gastric juice could be an important factor in this context, both by generating nitrite and by catalysing nitrosamine formation.

3236. Oral cancer in indulgent ladies

Bross, I. D. J. & Coombs, J. (1976). Early onset of oral cancer among women who drink and smoke. *Oncology* **33,** 136.

There have been a number of studies of the combined effects of drinking and smoking on oral cancer in men (Keller & Terris, *Am. J. publ. Hlth* 1965, **55,** 1578; Rothman & Keller, *J. chron. Dis.* 1972, **25,** 711), but these have not focused on age at onset of the disease. One difficulty is the shortage of male total abstainers for comparison. In the group of females considered in the paper cited above, there were enough abstainers to permit reliable comparisons of age at onset of both mouth and tongue cancer.

Both controls (1973 in all) and cancer patients (145 cases) were white women, categorized as abstainers, non-drinkers/smokers, light drinkers/non-smokers, light drinkers/smokers or heavy drinkers/smokers. Most smokers smoked one pack/day or less; light drinkers drank less than 30 bottles of beer or an equivalent amount of wine or spirit each month and heavy drinkers drank more than this. At first they were divided into two age groups, 40–64 and over 64 years.

The risk of oral cancer for light drinkers who were non-smokers was not significantly greater than that for the abstainers, but non-drinkers who smoked showed an age-adjusted relative risk of mouth cancer of 3·22, although they did not have a significantly increased risk of tongue cancer. Compared with the abstainers, light drinkers who smoked had a risk of 4·03 and heavy-drinking smokers a relative risk of 10·35 of getting mouth cancer. Results were similar for tongue cancer in these two groups. Thus the association between drinking, smoking and intra-oral cancer seems to be clearly established for the white females of this study. However, these age-adjusted relative risks are weighted over the risks from two age groups and are therefore 'diluted' by the older group.

To test the hypothesis that in women who drink and smoke the onset of cancer will occur at an earlier age, the indulgent groups were compared with older groups of abstainers. The age distribution of the cancer cases who were abstainers was shifted downwards relative to the other groups and the amount of shift needed to make the apparent risk of cancer in the indulgent groups no longer significantly different from that in the abstainers gave a measure of the shift in the age at onset of cancer.

The results showed that the risk of getting oral cancer remained significant after a 5-yr shift in the age of the abstainers for all drinking and smoking

groups but not for the non-smokers. For women who were light drinkers and smokers there was at least a 12·5-yr shift and for heavy drinkers who smoked there was more than a 20-yr shift in the age at onset of mouth cancer. Results were similar for women with tongue cancer except for the group who smoked but did not drink; they did not differ significantly from the abstainers.

It appears from this study that women who drink and smoke are at much greater risk of getting intra-oral cancer at younger ages than are abstainers. The authors warn that although in many cases it is necessary to use age adjustments, in situations where a shift in the age distribution of a disease is suspected, 'adjusting out' age can obscure real effects.

[*Contents continued*]

SHORT PAPER

Assessment of the acute toxicity and potential irritancy of hair dye constituents (*G. K. Lloyd, M. P. Liggett, S. R. Kynoch and R. E. Davies*) 607

MONOGRAPHS

Monographs on fragrance raw materials (*D. L. J. Opdyke*) 611

REVIEW SECTION

REVIEWS OF RECENT PUBLICATIONS 639

BOOK REVIEWS 641

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 645

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 649

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

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Index of Authors

The Index of Authors contains the names of all authors of original papers, review articles and Letters to the Editor. For Reviews of Recent Publications, Book Reviews and Abstracts, only the names of all the authors mentioned in the reference are given. For Articles of General Interest the names of all the authors mentioned in the text are provided, including those constituting the words *et al.*

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

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

Aalund, O. 246
Aarbakke, J. 364
Abbritti, G. 258
Abdellatif, A.M.M. 350
Abdel-Rahman, M.S. 256
Abraham, R. 347
Abrahamson, L.J. 401^{P}
Abrahamson, S. 75
Agersborg, H.P.K., Jr. 153
Aherne, F.X. 350
Ainsworth, L. 262
Albrecht, R. 352
Alderson, M. 471
Aldridge, W.N. 258
Alfrey, A.C. 82
Allen, J.R. 401^{P}, 419^{P}, 481
Alleva, F.R. 259
Alleva, J.J. 259
Al-Mossawi, M.A.J. 497
Al-Shahristani, H. 70
Ambrose, E.J. 344
Ambrosius, D. 275^{P}
Ames, B.N. 74, 645
Anderson, D. 483
Ansari, M.S. 71
Anver, M.R. 257
Aponte, C.J. 248
Arakaki, D.T. 647
Archer, M.C. 233^{R}
Arcos, J.C. 369
Argus, M.F. 369
Arsenault, G.P. 423^{P}
Ashby, J. 483
Astill, B.D. 167^{P}
Auty, R.M. 653
Aviado, D.M. 72, 239
Avila, R. 86
Axelsen, N.H. 246
Axelson, O. 480
Axford, A.T. 493

Bailin, P.L. 159
Balazs, T. 259
Balmer, M.F. 652
Barr, A. 249
Barr, W.T. 87
Barsotti, D.A. 401^{P}
Bartlett, B.E. 71
Bartsch, H. 66
Barzanti, V. 352
Battigelli, M.C. 84
Beale, M.G. 481
Beare-Rogers, J.L. 349, 350
Beatty, P.W. 481
Beaudoin, A.R. 257
Beck, F. 471
Beckwith, A.C. 495
Beevers, D.G. 480
Bell, J.M. 494
Bell, L. 645
Bellanca, N. 357
Bello, C.T. 480
Bellward, G.D. 252
Ben, T. 484
Benedetti, M.S. 646
Benhayem, S. 257
Benke, G.M. 309^{P}, 319^{P}
Berg, G.G. 70
Bergstrom, W.H. 249
Berman, W. 248
Berndt, W.O. 297^{P}
Bernstein, A. 255
Berry, H.K. 348
Bézard, J. 352
Bhaktaviziam, A. 247
Bhakthavathsalan, A. 78
Bhathal, P.S. 485
Biava, C.G. 69
Biemann, K. 423^{P}
Bigwood, E.J. 347
Bijster, G.M. 349, 350
Bilinski, E. 71
Bingle, A. 347
Bjeldanes, L.F. 77
Bjondahl, K. 445^{P}
Black, T.N. 86, 153, 533^{P}, 539^{P}
Blaha, G. 348
Blendis, L.M. 655
Blond, J.P. 352
Blumberg, P.M. 484
Blyth, C.A. 75
Bodine, S.R. 252
Boisseau, V.C. 248
Bollag, J.-M. 80
Bone, E.S. 655
Boniforti, L. 352
Bonse, G. 85
Boone, B.J. 253
Borchert, P. 645, 646
Borg, K. 350
Borzelleca, J.F. 153
Botticelli, A. 348
Botticelli, C.S. 348
Bouck, N. 484
Bouhuys, A. 653
Bowden, G.T. 484
Bowland, J.P. 350
Brada, Z. 485
Branch, R.A. 653
Brand, S. 261
Brantom, P.G. 379^{P}
Breslau, N.A. 647
Bridges, J.W. 75, 239
Brierley, J.B. 158
Briggs, W.A. 253
British Nutrition Foundation 641
Broillet, A.L. 646
Bross, I.D.J. 655
Brouwer, J.G. 646
Brouwers, J. 479
Brown, A.W. 158
Brown, C.A. 431^{P}
Brown, D. 260
Brown, M.M. 253
Brown, N.M. 309^{P}, 319^{P}
Brown, S.M. 654
Brunius, E. 349, 350
Brunner, R.L. 348
Bryce, F.R. 70
Buchet, J.-P. 479
Buhler, D.R. 353
Bui, T.-H. 478
Bulba, S. 485
Burg, A.W. 155
Burnett, C. 366
Burnett, C.M. 153
Burrows, R. 155
Busch, R.H. 82, 369
Buschbom, R.L. 213^{P}, 453^{P}
Bushell, C.T. 649
Butcher, B.T. 365
Butcher, F.R. 484
Butcher, R.E. 348
Butler, W.H. 70, 71, 481
Butterworth, K.R. 1^{P}, 383^{P}, 389^{P}, 503^{P}, 509^{P}, 523^{P}

Cain, J.A. 23^{P}
Calandra, J.C. 79, 337^{P}, 353, 354
Cali, G. 352
Calverley, J. 158
Campbell, B.C. 480
Cannon, W.C. 121^{P}, 213^{P}
Caperos, J. 160
Cappiello, V. 245
Cardona, R.A. 369
Carlton, W.W. 29^{P}, 121^{P}, 247, 411^{P}, 553^{P}, 563^{P}
Carman, M.J. 645
Carter, V.L., Jr. 73
Carver, B.D. 360
Casarett, L.J. 650
Casida, J.E. 75
Caspary, W.F. 248
Catanzaro, P.J. 251
Cavalleri, A. 244
Chan, S. 246
Chan, W.C. 143^{P}
Chandler, C. 39^{P}
Chandler, J.L.R. 69
Chang, F.C. 199^{P}

ห้องสมุด กรมวิทยาศาสตร์
- 7 พ.ย. 2521

Charbonneau, S.M. 70
Charles, J. 255
Charlton, K.M. 349, 350, 351, 352
Chasseaud, L.F. 239
Chaube, S. 479
Cheng, C.-K. 352
Chernoff, N. 360
Childs, E.A. 360
Ching, S.A. 650
Chiou, W.L. 73
Chiroff, R.T. 249
Choi, E. 74, 645
Christian, R.G. 349, 350
Christiansen, C. 248
Christopher, J.P. 93[P]
Chu, E.H.Y. 75
Chu, F.S. 199[P], 419[P]
Chudy, J.C. 547[P]
Chung, C.W. 325[P]
Chvasta, T.E. 155
Cianchetti, C. 258
Ciegler, A. 495
Ciofi-Luzzatto, A. 347
Cipolla, I.M. 352
Clandinin, D.R. 349, 351
Clark, D.E. 80
Clarkson, T.W. 70
Clayton, J.W. 85
Clouet, P. 352
Cocchi, M. 352
Colaianne, J.J. 354
Colburn, N. 484
Cole, P.V. 490
Coleman, G.L. 43[P]
Coli, C.A. 258
Collins, T.F.X. 86, 153, 154, 533[P], 539[P]
Collomb, M.H. 352
Colson, S.H. 354
Conaway, C.C. 482
Condliffe, P.G. 152
Conney, A.H. 75
Conti, L. 347
Cook, P.S. 357
Cooke, A.R. 155
Coombs, J. 655
Corbett, T.H. 257
Cornell, R.G. 257
Corner, A.H. 349, 350, 351, 352
Cornish, H.H. 156
Cosmides, G.J. 75
Cottrell, R.C. 17[P]
Couch, D.B. 251
Coulston, F. 347, 469
Couri, D. 256
Craig, D.K. 82
Crisley, F.D. 347
Crosby, N.T. 547[P]
Culbertson, J.C. 647
Cunningham, H.M. 101[P], 105[P], 183[P], 331[P]
Curradi, F. 258
Czaplicki, E.J. 80

Dalvi, R.R. 243
D'Amicis, A. 348
Darr, A.G. 35[P]
Davey, K. 349
Davies, D.J.G. 654
Davies, R.E. 55[P], 265[L], 607[P]
Davies, N.D. 246
Davison, K.L. 71
Dean, R.T. 343
Dedrick, R.L. 152
DeFonso, L.R. 245
de Gubareff, T. 347
Deguia, E. 248
De Jesus, P.V. 258
Deknudt, Gh. 158
Del Carmine, R. 352
Department of Employment 63
Department of Industry 237
Department of the Environment 467
Derache, R. 147[P], 352
De Rosa, F. 258
Desai, D.B. 493
de Saint Blanquat, G. 147[P]
de Serres, F.J. 74
Deutsche Forschungsgemeinschaft—Kommission für Pflanzenschutz-, Pflanzenbehandlungs- und Vorratsschutzmittel 641
Diamond, L. 39[P]
Dick, C.E. 69
Diener, U.L. 246
Dilley, J.V. 73
di Mayorca, G. 484
Dingle, J.T. 343
DiPaolo, J.A. 75
Djupsund, B.M. 160
Docks, E.L. 161
Dodds, T.C. 240
Doerr, R.C. 441[P]
Doherty, D. 75
Doherty, R.E. 72
Doige, C.E. 494
Donaldson, B. 79
Donovan, P.J. 75
Dooley, C.J. 441[P]
Dorling, P.R. 485
Dorough, H.W. 469
Douglas, D.R. 260
Downie, R.H. 70
Drake, J.J.-P. 503[P]
Draser, B.S. 370
Dresser, D.W. 240
Dressler, I.A. 353, 354
Drew, R. 485
Drew, R.T. 72, 73, 245
Driedger, P.E. 484
Drinkwater, N.R. 646
Drotman, R.B. 309[P]
Dubini, M. 487
Dueck, J.W. 495
Dukes, M.N.G. 241
Duncan, J.H. 109[P]
Dünger, M. 275[P]

Eagle, E. 153
Ebert, A.G. 153
Edwards, J.H. 255
Edwards, P.M. 154, 155
Eisenbrand, G. 500
Eldefrawi, M.E. 257
Elinder, C.-G. 479
Elling, F. 246, 247
Elliot, L.A. 348
Ellis, G.P. 472
Emmerling, D. 497
Emrich, D. 248
Endres, J. 257
Engfeldt, B. 349, 350
Englund, P. 246
Ernster, L. 351
Ershoff, B.H. 358
Estabrook, R.W. 75
Evans, J.G. 523[P]
Evarts, R.P. 139[P], 431[P]
Eyl, T.B. 70

Fahim, M.S. 488
Fahim, Z. 488
Fan, T.Y. 423[P]
Federal Register 479
Feigin, R.D. 347
Fell, G.S. 479
Fennell, D.I. (The late) 411[P]
Fernandez, J. 160
Feron, V.J. 491
Fiddler, W. 441[P]
Filer, L.J., Jr. 347
Fine, D.H. 423[P]
Fiorentino, E. 352
Fisher, D.R. 362
Fitzgerald-Finch, O.P. 479
Fitzhugh, O.G. 646
Flowers, N.C. 72
Fong, Y.Y. 143[P]
Food Chemical News 153
Forbes, P.D. 55[P], 265[L]
Ford, E.J.H. 243, 244
Forni, A. 652
Foster, T. 39[P]
Foulds, L. 151
Fourie, D.T. 162
Fouts, J.R. 75
Fowler, B.A. 253
Fox, B.W. 471
Fraumeni, J.F., Jr. 344
Freedman, R.B. 75
Fremlin, J.H. 480
Freudenthal, R.I. 497
Freundt, K.T. 243, 244
Friberg, L. 479
Friedman, M.A. 251, 252
Friend, D.W. 350, 351
Fries, G.F. 482
Fritsch, P. 147[P]
Fry, R.J.M. 93[P]
Fukushima, M. 478, 479
Furia, T.E. 357
Furukawa, K. 251

Gabe, M. 643
Gaillard, D. 352
Gaines, T.B. 153
Gak, J.-C. 649
Galli, C. 156
Gangolli, S.D. 383[P], 389[P], 509[P]
Gardiner, J.S. 493
Gardner, D.L. 240
Garfinkel, J. 654
Gatti, G.L. 350, 351, 352
Gaunt, I.F. 1[P], 379[P], 383[P], 389[P], 503[P], 509[P], 523[P]
Gavrilenko, E.V. 153
Gaylor, D.W. 153
Gehring, P.J. 482
Gentry, R.P. 71
Gershbein, L.L. 131[P], 173[P]
Gibson, T.P. 253
Giles, A.L., Jr. 325[P]
Gilka, F. 350, 351
Gillette, J.R. 75
Gilman, A. 241
Gingell, R. 157
Ginocchio, A.V. 1[P]
Giovanoli-Jakubczak, T. 70

Glauser, E.M. 480
Glauser, S.C. 480
Glover, E. 482, 483
Gloxhuber, Chr. 496
Goff, U. 423[P]
Golberg, L. 347
Gold, M. 257
Goldberg, A. 480
Goldenthal, E.I. 366
Goldschmidt, B.M. 246
Gonatas, N.K. 258
Goodall, C.M. 499
Goodman, L.S. 241
Gopinath, C. 243, 244
Goto, I. 154
Goto, K. 78
Gough, T.A. 437[P]
Gould, L.V. 249
Goyer, R.A. 253
Graham, B. 85
Graham, D.I. 258
Graillot, C. 649
Gralla, E.J. 43[P]
Gram, T.E. 87, 497
Grasso, P. 1[P], 389[P], 503[P], 509[P], 523[P]
Gray, G.C. 153
Gray, T.J.B. 389[P]
Greb, W. 496
Greig, J.B. 481, 482
Greim, H. 85
Gribbin, J. 74
Griffaton, G. 352
Griffiths, K. 87
Gross, J. 160
Groth, D.H. 261
Guberan, E. 160
Guenter, W. 71
Gunther, F.A. 67, 341, 641
Gutenmann, W.H. 157
Guttman, S. 39[P]
Gyrd-Hansen, N. 246

Hackney, J. 484
Haddad, J.G., Jr. 248
Hagen, E.C. 646
Hagino, N. 478, 479
Hahn, T.J. 248
Hald, B. 246, 247
Hall, D.G. 488
Hall, R.L. 457[R]
Hamilton, P.B. 247
Hand, C. 72
Hanig, J.P. 35[P], 354
Hanoune, J. 354
Hansen, E.V. 364
Hansen, W.H. 646
Hardin, R.T. 349, 350, 351
Hardy, J. 379[P], 383[P]
Harris, E.S. 73
Harris, S.B. 366
Harvey, T.C. 480
Harwig, J. 495
Haseman, J.K. 483
Hawthorne, V.M. 480
Hay, A. 481
Hay, I.T. 162
Hayes, A.W. 23[P]
Hearnsberger, A.P. 359
Heggtveit, H.A. 349, 350
Heidelberger, C. 484
Heijkenskjöld, L. 351
Henderson, R.G. 248
Henderson, W.J. 87
Hendin, B.A. 248
Hennings, H. 484
Henschler, D. 85
Hernberg, S. 244
Hertzog, P.J. 485
Hesch, R.D. 248
Hetland, L.B. 256
Hierlihy, L. 80
Hiles, R.A. 205[P]
Hill, M.J. 655
Hinsman, E.J. 247
Ho, O.L. 347
Hobart, M.J. 240
Hobbs, E.J. 79
Hoben, H.J. 650
Hodges, N.D.M. 654
Hoffmann, K.A. 365
Hogan, M.D. 159
Hohenleitner, F.J. 257
Hollaender, A. 74
Hollander, H. 160
Hollins, J.G. 70
Holm, S. 335[P]
Holmes, P.A. 77
Holscher, M.A. 481
Holson, J.F., Jr. 153
Holtzman, E. 344
Hook, G.E.R. 483
Horan, L.G. 72
Horning, M.G. 645
Houtsmiller, M.T. 351, 352
Hoyer, S. 254
Hruby, R. 595[P]
Hsia, M.-T.S. 419[P]
Huff, W.E. 247
Hughes, R.E. 358
Hulan, H.W. 349, 350
Hummel, R.A. 482
Humphries, W.R. 489
Hunt, J.R. 350, 351, 352
Hunt, P.F. 360
Hunter, A.L. 243
Husain, F.E.R. 479
Hutchinson, D.L. 69
Hutzinger, O. 255
Hyldgaard-Jensen, J. 246

Ide, H. 251
Igisu, H. 154
Inoue, K. 367
Inouye, M. 447[P]
International Agency for Research on Cancer 237
Ippen, H. 496
Ishizaki, A. 478, 479
Isomaa, B. 160
Ito, Y. 367
Iverson, F. 70
Iwatsuki, K. 83
Iyengar, J.R. 79
Izumi, K. 154

Jackson, L.K. 495
Jackson, W.T. 481
Jacobsen, Chr. 247
James, L.F. 366
Jamieson, G.A. 642
Jasmin, G. 370
Jenner, P.M. 646
Johnson, B. 494
Johnson, J.H. 80
Johnstone, R.A.W. 643
Joint FAO/WHO Expert Committee on Food Additives 639
Jonas, R.E.E. 71
Jones, A. 74
Jones, B. 255
Jones, D.J. 255
Jones, G. 481
Jones, J.D. 482, 494
Jones, R. 159
Jones, W.I. 646
Jönsson, L. 87
Jordan, W.H. 29[P]
Jordö, L. 259
Jucker, E. 72

Kaehny, W.D. 82
Kapp, R. 366
Karl, M.M. 481
Kato, M. 154
Kattermann, R. 248
Katz, S.A. 83
Kauppi, M. 575[P]
Kawamura, Y. 154
Kean, E.A. 494
Keefe, R.T. 135[P]
Keeler, H.V. 153
Keeler, R.F. 260
Kelsey, W. 484
Kelton, S.C., Jr. 245
Kendall, F.M. 257
Kende, A. 483
Kennedy, G.L., Jr. 153, 337[P], 353, 354
Kennedy, T.H. 499
Kensler, C.J. 155
Kent, T.H. 361
Keplinger, M.L. 79, 153, 337[P], 353, 354
Kesäniemi, Y.A. 77
Khanna, K.L. 156
Khera, K.S. 61[P], 80, 157, 487
Kilgore, L.T. 359
Kimelberg, H.K. 484
Kimmerle, G. 488
King, C.M. 369
King, L.J. 73
Kinoshita, F.K. 153, 337[P]
Kirschman, J.C. 77, 153
Kiss, I.S. 383[P]
Kjellström, T. 479, 480
Klaassen, C.D. 496
Klein, R.M. 248
Kleinekoort, J.S.W. 349, 350
Knaap, A.G.A.C. 69
Kodama, R. 251
Kolonel, L.N. 479, 480
Koransky, W. 80
Koropatnick, D.J. 74
Korte, F. 469
Koschier, F.J. 297[P]
Koss, G. 80
Kozuka, Y. 247
Kramer, J.K.G. 349, 350, 351, 352
Kramer, L.L. 159
Kramers, P.G.N. 69, 496
Kreula, M. 575[P]
Kriek, N.P.J. 579[P]
Krishna, G. 161, 262
Krogh, P. 246, 247
Kromer, W. 243
Krop, S. 35[P], 354
Krüger, G. 254
Kruysse, A. 491

Kuhr, R.J. 469
Kumaki, K. 483
Kuratsune, M. 195[P]
Kuroiwa, Y. 154
Kuschner, M. 245
Kynoch, S.R. 607[P]

Lakshminarayan, S. 479
Lal, H. 364
Lalich, J.J. 401[P]
Lam, P. 74
Lamont, A. 479
Lamperti, A. 348
Lancet 74
Landin, W.E. 257
Lane, B.P. 254
Larsen, A.E. 246
Larsen, J. Chr. 364, 487
Larsson, K.S. 494
Laskin, S. 245
Latham, A.N. 247
Lauwerys, R.R. 479
Lawrence, G.A. 101[P], 105[P], 183[P], 331[P]
Leach, L.J. 652
Leary, H.L., Jr. 368
Leber, P. 497
Lecce, J.G. 368
Lech, J.J. 253
Ledingham, J.G.G. 248
Lee, K.P. 491
Lefevre, P.A. 483
LeGendre, G.R. 82
Lemarchal, P. 352
Lembach, K.J. 481
Lemkey-Johnston, N. 347
Léonard, A. 158
Le Page, R.N. 485
Le Quesne, P.M. 493
Lerner, D. 159
Lewis, E.B. 75
Ley, H.L. 69
Lichti, U. 484
Lieberwirth, K. 244
Liedén, S.Å. 494
Liggett, M.P. 607[P]
Lijinsky, W. 229[P], 269[P], 500
Lind, B. 479, 480
Lindsten, J. 478
Lindström, E. 7[P], 289[P]
Linnman, L. 479
Lisk, D.J. 157, 257
Litewka, B. 491
Litterst, C.L. 87, 497
Liu, M. 78
Livingston, S. 248
Lloyd, A.G. 509[P]
Lloyd, G.K. 607[P]
Lloyd, J.B. 471
Lo, L.W. 74
Lock, E.A. 258, 363
Lockhart, W.L. 85
Loew, F.M. 494
Long, E.L. 646
Longstaff, E. 483
Lorenz, P. 496
Lorke, D. 69
Lourenço, A.G. 86
Lowrance, W.W. 66
Lowy, R. 352
Lucas, J. 65
Lucier, G.W. 483
Luginbühl, H. 7[P], 289[P]
Lund, M. 248

McCann, J. 74, 645
McConnell, I. 240
McDaniel, O.S. 483
McDonald, J.E. 131[P]
MacDonald, W.C. 495
McGregor, D.B. 262
MacGregor, J.T. 225[P]
Machemer, L. 69
McKerrow, C.B. 493
McKirdy, A. 479
McLaughlin, J. 153, 154
McLellan, J.S. 480
McMartin, K.E. 256
McPhail, M.F. 437[P]
McPherson, F. 75
Madsen, A. 246
Maenza, R.M. 370
Magos, L. 70, 71
Mahadevan, S. 349, 350, 351, 352
Mahr, U. 359
Maibach, H.I. 496
Maier, P. 647
Makar, A.B. 256
Mäkinen, S.M. 575[P]
Makino, F. 81
Makowski, P. 78
Malling, H.V. 74
Mallov, J.S. 159
Malnoë, A. 646
Manara, L. 487
Manchon, Ph. 352
Mann, L.I. 78
Mannering, G.J. 75
Manney, T.R. 74
Manns, J.G. 494
Maranesi, M. 352
Marasas, W.F.O. 579[P]
March, B.E. 71
Marchisio, M.A. 487
Mariani, A. 347, 348
Marquardt, H. 97[P]
Marrow, G.S. 482
Martin, A.G. 256
Martin, D.J. 650
Martinsson, K. 87
Mass, M.J. 254
Masuda, F. 367
Masuda, Y. 195[P]
Matlaga, B.F. 498
Matsumura, F. 482
Matsunaga, K. 72
Matte, R. 248
Matter, B. 647
Mavier, P. 354
Mayhew, E. 484
Mays, C.W. 362
Medlinsky, H.-L. 248
Meisner, H. 246
Melancon, M.J., Jr. 253
Melville-Jones, C. 87
Mennini, T. 487
Meyer, T. 364, 487
Michalek, H. 350, 351, 352
Miles, W.F. 79
Millbank, L. 247
Miller, E.C. 645, 646
Miller, J.A. 645, 646
Miller, W.J. 71
Mills, E.A. 479
Milne, J. 261
Miltenburger, H.G. 359
Mimnaugh, E.G. 87, 497
Minard, R.D. 80
Mitchell, C.A. 84
Moffatt, R.E. 159
Mollenhauer, H.H. 80
Møller, T. 246
Mondal, S. 484
Montesano, R. 66
Moore, B.G. 23[P]
Moore, J.A. 482, 483
Moore, M.R. 480
More, K.N. 493
Morgan, W.D. 480
Morgan, W.K.C. 479
Morrison, J.M., Jr. 35[P], 354
Morrison, J.N. 489
Mortensen, H.P. 246
Mortimer, R.K. 74
Moss, S.H. 654
Mulligan, L.T. 167[P]
Munro, I.C. 70
Murakami, U. 447[P]
Murphy, J.C. 86
Murray, D. 85
Myers, T. 135[P]

Nagayama, J. 195[P]
Nakaue, H.S. 353
Nakazawa, N. 81
Narisawa, T. 262
Nash, L. 361
National Academy of Sciences 151
Nayfield, S.G. 361
Neal, R.A. 243, 481
Neathery, M.W. 71
Nebert, D.W. 483
Nelson, A.A. 646
Nelson, N. 245
Nelson, R.L. 75
Nemeroff, C.B. 347
Nera, E.A. 349, 350
Nettesheim, P. 88
Netz, H. 244
Neurath, G.B. 275[P]
Newberne, P.M. 135[P]
Newsome, W.H. 361
Ng, R. 650
Niazi, S. 73
Nieminen, L. 445[P]
Nishimura, H. 152, 479
Niwa, A. 483
Nixon, J.E. 283[P]
Noda, K. 251
Nogawa, K. 478, 479
Norback, D.H. 481
Nordberg, G.F. 65, 478
Norman, A.W. 248
Norseth, T. 70
Nurminen, M. 244

Official Journal of the European Communities 352
Ohenoja, E. 445[P]
Ojanen, H. 445[P]
Okada, S. 81
Olive, C.W. 369
Oliver, D.O. 248
Oliver, R.M. 481
Olney, J.W. 347
Olson, R.J. 82, 369
Olsson, R. 259
Opdyke, D.L.J. 611[M]

Orton, T.C. 483
Oser, B.L. 69, 457[R]
Osterberg, R.E. 86
Ottoway, J.M. 479
Oyen, F. 490

Palese, M. 256
Pande, S.V. 352
Parke, D.V. 73, 75
Parry, J.M. 497
Parry Jones, A. 493
Pass, D.A. 71
Paterson, P.J. 479
Patterson, E. 484
Paul, C. 70
Pauli, L.L. 248
Paulson, G.W. 492
Pein, F.G. 275[P]
Pensabene, J.W. 441[P]
Peraino, C. 93[P]
Perek, M. 348
Perticoni, G.F. 258
Petersen, O.K. 246
Petrelli, M.P. 248
Petty, T.L. 479
Pevny, I. 84
Pfaender, F.K. 353
Pheasant, A.E. 261
Phillips, J.C. 17[P], 383[P]
Pierce, E.C. 153
Pietropaolo, C. 352
Pimentel, J.C. 86
Pipy, B. 352
Pisano, L. 352
Piscator, M. 479, 480
Pitkin, R.M. 347
Pitot, H.C. 646
Plaa, G.L. 485
Pleasure, D.E. 258
Plummer, F.R. 71
Plunkett, E.R. 642
Pohl, L.R. 262
Pohlmann, E. 244
Poland, A. 482, 483
Popp, J.A. 484
Popper, H. 342
Potokar, M. 496
Preston, C. 248
Pretanik, J.S. 360
Preussmann, R. 500
Prew, J.H. 533[P], 539[P]
Price, J.M. 69
Priestly, B.G. 485
Pritchard, A.B. 77
Purchase, I.F.H. 483

Quarterman, J. 489

Rabin, B.R. 75
Radwan, Z. 85
Raman, P.T. 247
Ramirez, A. 72
Ramsey, J.C. 482
Rancitelli, L.A. 589[P]
Rankin, P.W. 187[P]
Rao, G.S. 156
Ravnskov, U. 246
Reading, J.C. 479
Reagan, R.L. 87
Reddy, B.G. 262
Regan, J.W. 77
Reichert, D. 85
Reiss, J. 260, 303[P]
Renwick, A.G. 370
Reuter, J. 160
Reynolds, W.A. 347
Rhee, V. 347
Rhyne, B.C. 253
Richards, D.E. 261
Richens, A. 247, 470
Richter, W.R. 353, 354
Rieger, K. 160
Riopelle, J.L. 370
Ritschel, W.A. 365
Ritter, H. 248
Roberts, G. 487
Robinson, D.M. 642
Robinson, G.R. 354
Robinzon, B. 348
Robson, A.M. 481
Rocquelin, G. 351
Rødbro, P. 248
Rodman, N.F. 361
Roe, F.J.C. 344
Roels, H.A. 479
Roenigk, H.H., Jr. 159
Rogers, R.W. 359
Roobol, A. 75
Rose, J.Q. 482
Rosenkranz, H.S. 252
Rossow, R.W. 484
Rostgaard, M. 246
Rowe, D.J.F. 247, 248
Rowe, V.K. 490
Rowland, I.R. 17[P]
Rowland, J. 499
Rowland, J.C. 601[P]
Rowland, S. 74
Roxburgh, S. 479
Ruddell, W.S.J. 655
Ruddick, J.A. 80, 157, 361, 495
Rufino, F. 97[P]
Ruggles, D.I. 153
Ruoslahti, E. 484
Russell, L.A. 601[P]
Russell, R.G.G. 248
Rutqvist, L. 246
Ruzo, L.O. 255

Safe, S. 255
Sainani, G.S. 493
St. John, L.E., Jr. 257
Salthouse, T.N. 498
Salvaggio, J.E. 365
Salzman, L.K. 357
Samitz, M.H. 83
San, R.H.C. 74, 75, 484
Sanders, P.R. 365
Sanders, V. 252
Sansing, G.A. 29[P], 246, 553[P]
Sarver, J.W. 491
Satoh, T. 247
Sauer, F.D. 349, 350, 351, 352
Sawicki, J.E. 251
Scala, R.A. 153
Schaffner, F. 342
Sharp, C.R. 248
Schaumburg, H.H. 492
Scheel, L.D. 261
Scheline, R.R. 364, 487, 645
Schmähl, D. 475, 500
Schmid, W. 75, 647
Schniepp, M.L. 69
Schoenberg, J.B. 84
Schreiber, O. 275[P]
Schulz, K.H. 496
Schumacher, H.J. 153
Schumacher, R. 257
Scientific Committee for Food, Commission of the European Communities 352
Scott, P.M. 495
Scott, R. 479
Seal, R.M.E. 255
Seaman, S. 79
Searcy, G.P. 494
Seaton, A. 255
Seidman, I. 246
Seinen, W. 81
Sell, J.L. 71
Sellakumar, A. 499
Selvin, S. 654
Semprini, M.E. 347, 348
Sen, N.P. 79
Seńczuk, W. 491
Sergiel, J.P. 351
Serra, G. 487
Shah, H.C. 364
Sharpe, G.L. 494
Sharpe, L.G. 347
Shearer, W.T. 481
Sherwood, M. 74
Shibata, I. 478, 479
Shihab, K.M. 70
Shimkin, M.B. 651
Short, D.D. 109[P], 117[P]
Shtenberg, A.I. 153
Sifri, M. 71
Silverman, A.P. 85
Simaan, J. 239
Simpson, P.J. 490
Sims, J.L. 353
Sippel, H.W. 77
Siracusa, A. 258
Sjö, O. 248
Skadhauge, E. 247
Skelton, F.S. 485
Skerfving, S. 70
Slaga, T.J. 484
Small, D.G. 248
Small, H. 70
Smith, D. 363
Smith, D.G. 72
Smith, F.A. 652
Smith, J.M. 242
Smith, R. 248
Smith, S.H. 153, 337[P], 354
Smith, T.J. 479
Snapir, N. 348
Snyder, D.R. 43[P]
Sobels, F.H. 69, 75
Solberg, L.A. 362
Solheim, E. 645
Söling, H.D. 342
Sonawane, B.R. 483
Sonders, R.C. 69
Song, L. 423[P]
Soong, R. 71
Spagnuolo, C. 156
Spano, P.F. 156
Speizer, F.E. 72
Spencer, P.S. 492
Stadel, B.V. 162
Staffeldt, E. 93[P]
Stake, P.E. 71
Stallknecht, G.F. 260
Stamp, T.C.B. 248
Stegint, L.D. 347

Stein, M.E. 155
Steinfeld, J. 69
Stenbäck, F. 499
Stenbäck, F.G. 601^P
Stengel, D. 354
Stevenson, D.E. 360
Steyn, M. 579^P
Steyn, P.S. 579^P
Stich, H.F. 74, 75, 484
Stillwell, W.G. 645
Stoner, G.D. 651
Strattan, C.E. 109^P
Strausburg, J. 366
Struyk, C.B. 351, 352
Styles, J.A. 483
Sugiyama, T. 78
Sumner, A.J. 154
Sun, P.S. 419^P
Sunderman, F.W., Jr. 370
Sunshine, G. 75
Surak, J.G. 187^P
Suzuki, S. 247
Suzuki, Y. 367
Svendsen, C. 247
Swahn, O. 246
Swart, J. 347
Swineyard, C.A. 479
Syrotuck, J. 368
Szczech, G.M. 247
Szwabowska, M. 257
Szyszka, K. 156

Tager, J.M. 342
Tanimura, T. 152
Tan, H.S. 365
Tanaka, K. 494
Tanner, T.M. 213^P, 453^P
Tansy, M.F. 257
Taori, G.M. 247
Tavernese, G. 352
Taylor, D.M. 482
Taylor, G.J. 72, 73
Taylor, G.N. 362
Taylor, H.W. 88, 229^P, 269^P, 500
Teorell, T. 152
Tephly, T.R. 256
Terry, G. 487
Terry, L.S. 651
Thacker, H.L. 553^P, 563^P
Thomas, B.J. 480
Thomas, D.J. 254
Thompson, N.P. 187^P
Thompson, T.L. 651
Thorpe, E. 360
Tiitola, K. 244
Timmer, W.G. 349, 350
Tindall, J.P. 159
Tolonen, M. 244
Tolos, W.P. 261
Tomatis, L. 66
Torjussen, W. 362
Torkelson, T.R. 490
Trenholm, H.L. 70
Trentini, G.P. 348
Trivett, G. 487
Trosko, J.E. 75, 484
Troxell, M.C. 651
Truhaut, R. 649
Tuite, J. 411^P
Turchetto, E. 352
Turner, J.H. 69
Tweats, D.J. 497

Uchiyama, M. 367
Uenaka, H. 78
Uhlich, M. 84
Ulander, A. 480
Ulsamer, A.G. 239, 354
Ungar, H. 484
University of Texas System Cancer Center 470
Urbach, F. 55^P, 265^L

Vaheri, A. 484
Valic, F. 653
van der Beek, A. 351, 352
Van Duuren, B.L. 246
VanErt, M. 84
Van Kampen, K.R. 366
Van Miller, J.P. 401^P, 481
van Rensburg, S.J. 579^P
Varkey, K. 247
Venitt, S. 649
Vesey, C.J. 490
Vigliani, E.C. 652
Villareale, M. 249
Virgo, B.B. 252
Vles, R.O. 349, 350
Voelker, R. 366
Vogel, E. 69, 75
Vogin, E.E. 69
Vogtmann, H. 349, 350, 351
von Ledebur, M. 647
Vos, J.G. 482
Vuataz, L. 7^P, 289^P

Wagemann, R. 85
Wagstaff, D.J. 354
Wahlberg, J.E. 489
Walker, A.I.T. 360
Walsh, M.J. 309^P
Walters, C.L. 655
Walton, R.J. 248
Waring, R.H. 261
Warner, G.T. 248
Wasserman, R.H. 249
Watanabe, T. 72
Watson, C.R. 121^P
Waylonis, G.W. 492
Wazeter, F.X. 366
Webb, K.S. 437^P
Webb, W.K. 646
Wedig, J.H. 43^P
Wegman, D.H. 72
Wehner, A.P. 82, 121^P, 213^P, 369, 453^P, 589^P
Weigland, W. 160
Weill, H. 365
Weinhardt, F. 254
Weisburger, E.K. 139^P
Weisburger, J.H. 97^P, 262
Wenk, M.L. 139^P
Wenner, C.E. 484
Wentzler, T.H. 482
Werner, E. 155
Werry, J.S. 357
West, G.B. 472
Westwood, F.R. 483
Whalen, G. 487
WHO Study Group 63
Wiegand, R.G. 69
Wilkerson, C.L. 213^P, 589^P
Williams, D.J. 75
Williams, D.T. 80
Williams, H. 85
Williamson, J.R. 342
Willems, M.I. 81
Willes, R.F. 70
Wishnok, J.S. 233^R
Wislocki, P.G. 645, 646
Witschi, H. 485
Wlodkowski, T.J. 252
Wolf, C.R. 73
Wong, C.-Q. 262
Woodard, J.C. 109^P, 117^P
Woodford, F.P. 470
Woodhill, J.M. 357
Worthington, B.S. 368
Wright, E. 358
Würzner, H.-P. 7^P, 289^P
Wyatt, R.D. 247

Yager, J.D. 484
Yamasaki, E. 74, 645
Yamazaki, M. 247
Yano, T. 251
Yasenchak, L.P. 498
Yasuda, I. 83
Yates, A.J. 479
Yoshida, T. 367
Young, S. 260
Younger, R.L. 80
Yuile, C.L. 652
Yuspa, S.H. 484

Zaalberg, J. 349, 350
Zakhari, S. 239
Zapp, J.A., Jr. 491
Ziller, S.A. 49^P
Zimmermann, J.L. 411^P
Zinkl, J.G. 482
Ziskind, M.M. 365
Žuškin, E. 653
Zwicker, G.M. 121^P

Fd Cosmet. Toxicol. Vol. 15, pp. 9–16. Pergamon Press 1977. Printed in Great Britain

Index of Subjects*

Acetaldehyde, high blood levels in rats and man after simultaneous ethanol ingestion and carbon disulphide inhalation 244; placental and foetal metabolism in rats 77

Acetaldehyde *N*-methyl-*N*-formylhydrazone (see Ethylidene gyromitrin)

Acetamide, *N*-hydroxy-*N*-2-fluorenyl- and *N*-hydroxy-*N*-4-biphenyl-, covalent binding with nucleic acid effected by human acyltransferases 369

2-Acetylaminofluorene, tumour induction in rat liver following ingestion of, enhanced by phenobarbitone or BHT 93^{P}

Ackee, biochemical study of cases of Jamaican vomiting sickness caused by ingestion of the unripe fruit 494

Acrylamide, neurotoxic effects in animals and man 154; reproduction study in rats 154

Acrylonitrile, determination in foods by headspace GLC 547^{P}; lung lesions in guinea-pigs exposed to high dust concentrations of acrylic fibre 86

Adhesives, spray-type, no resorption increase or malformations in offspring after inhalation of toxic doses by pregnant hamsters 86

Adriamycin, positive results in micronucleus test 647

Aflatoxin, B_1, alone and with rubratoxin B, acute oral effects in infant rats 23^{P}; book review on IARC monograph on carcinogenic risks from 237

Albumin, no detectable effect on tin absorption in rats dosed with stannous chloride 147^{P}

Alcoholic congeners, effects on ethanol elimination by isolated perfused rat liver 653

Alkylbenzene sulphonates, linear, effect on mouse foetuses of dermal or sc administration of detergent formulation containing 367

Alkyl polyethoxylates (see under Polyoxyethylene condensates)

***p*-Allylanisole** (see Estragole)

Allyl esters, properties, use as fragrance raw material, status, toxicity and metabolism of: *n*-butyrate 613^{M}, cinnamate 615^{M}, cyclohexylacetate 617^{M}, heptylate 619^{M}, phenylacetate 621^{M}

Aluminium, high tissue levels tentatively associated with encephalopathy in dialysis patients treated for serum-phosphorus control with 82

Aluminium chlorhydrate, no skin changes in human topical application study 160; no toxic effects and low penetration in inhalation studies in animals 160

Amaranth, effect of low-fibre rat diet on toxicity of high intake of 358; reduction by whole-cell extracts of contents of rat caecum 487; review of recent reports on teratology of 153; serum levels, metabolism and excretion after administration to rats in diet or drinking-water or by gavage 77; teratogenicity study in cats 487

Amines (see also specific compounds), primary and secondary, identified by GLC in vegetables, pickles, fish, bread, cheese, stimulants, animal feeds and surface waters 275^{P}

Aminoazobenzene, 3′-methyl-4-*N*-dimethyl-, effect of a tryptophan supplement on liver-tumour incidence in rats fed diet containing 431^{P}

4-Aminobenzoic acid, and UV irradiation, combined effects on bacterial DNA 654

***N*$^{\epsilon}$-(DL-2-Amino-2-carboxyethyl)-L-lysine** (see Lysinoalanine)

4-Amino-2-hydroxytoluene, skin and eye irritancy in rabbits and acute oral toxicity in rats 607^{P}

Aminonitrophenols, 2-amino-4-nitro-, skin and eye irritancy in rabbits and acute oral toxicity in rats 607^{P}; 4-amino-2-nitro-, lifetime skin application studies in mice and rabbits 601^{P}

Aminophenol, 3- and 4-, skin and eye irritancy in rabbits and acute oral toxicity in rats 607^{P}

Analytical chemistry, book review on: survey of recent literature on mass spectrometry 643, techniques of liquid scintillation spectrometry 471

Anise oil, liver regeneration in partially hepatectomized rats increased by 173^{P}

Anisole, 4-allyl- and 4-propenyl-, liver regeneration in partially hepatectomized rats increased by 173^{P}

ANIT (see α-Naphthylisothiocyanate)

Anticonvulsants (see also specific compounds), disturbance of calcium and vitamin D metabolism in long-term therapy with 247

Antioxidants (see also specific compounds), development of polymeric relatively non-absorbable phenols or substituted phenols for food use 357; FAO/WHO toxicological evaluation 639

Antiperspirants (see also specific compounds), ventilatory function tests in man, following axillary application of aerosol product 39^{P}

Arsenate, sodium, ultrastructural and biochemical effects in cells of renal proximal tubules of rats after prolonged ingestion of drinking-water containing 253

Ascorbic acid, no abortifacient or other adverse effects of oral administration to pregnant hamsters, rats and guinea-pigs 259; no detectable effect on tin absorption in rats dosed with stannous chloride 147^{P}; sodium salt, inhibition of nitrosopyrrolidine formation in frying bacon by 79

L-Ascorbyl palmitate, inhibition of nitrosopyrrolidine formation in frying bacon by 79

Azinphos-methyl, 12-wk inhalation study in rats 488

Azo dyes, sulphonated, reduction by whole-cell extracts of contents of rat caecum 487

Azoxymethane, BHT inhibition of intestinal-tumour induction by sc injections of 139^{P}; liver haemangiosarcomas but no colonic tumours induced in guinea-pigs by 262

Azulenes, liver regeneration in partially hepatectomized rats increased by sc injection of 173^{P}

Bacteria, of gut, *in vivo* methaemoglobin formation in rats resulting from nitrobenzene reduction by 262; of rat caecum, reduction of sulphonated azo dyes by 487

Benomyl, and hydrolysis products, effects on *Tetrahymena pyriformis* 187^{P}

Benzene, incidence of leukaemia in workers exposed to, and possible mechanism of leukaemogenic action of 652

Benzo[*a*]pyrene, effect of intratracheal instillation of various dusts on respiratory-tumour induction by 499; probable mechanism for transport to target organ in lower gut of rats 370

Biphenyl, metabolism and urinary excretion of phenolic derivatives in rat and pig 364

4,4′-Bis-([4-anilino-6-(*N*-methyl-*N*-2-hydroxyethylamino)-1,3,5-triazin-2-yl]amino)stilbene-2,2′-disulphonic acid, disodium salt, negative results in Ames tests 262

4,4′-Bis-([4-anilino-6-morpholino-1,2,5-triazin-2-yl]amino)-stilbene-2,2′-disulphonic acid, disodium salt, negative results in Ames tests 262

*The significance of the superscripts P, M, R and L is given in paragraph 2 of the Index of Authors (p. 3).

Bismuth, in skin creams, cases of brain disturbance attributed to interference with cerebral oxidative metabolism by 254

4,4′-Bis-(2-sulphostyryl)biphenyl, disodium salt, negative results in Ames tests 262

Black PN, long-term toxicity study in mice 503[P]

Bleomycin, negative results in micronucleus test 647

Bread, occurrence of sterigmatocystin on various types of 260; variation in citrinin production by *Penicillium chrysogenum* on various types of 303[P]

Bromobenzenes, metabolism of mono- to pentabromo compounds in rabbit, some evidence for epoxide intermediates 255

5-Bromo-5-nitro-1,3-dioxan, as cosmetics preservatives, acute oral and ip and subacute oral tests in rats and skin tests in rabbits 496

Bronidox (see 5-Bromo-5-nitro-1,3-dioxan)

Brown FK, and constituents, mutagenicity studies in *Salmonella typhimurium* 649

***n*-Butanol,** as congener in alcoholic drinks, effect on ethanol elimination by isolated perfused rat liver 653

2-Butanone (see Methyl ethyl ketone)

Butylated hydroxyanisole, no observed effects on intragastric formation of *N*-nitrosamine from nitrite and dimethylamine 167[P]

Butylated hydroxytoluene, 2-acetylaminofluorene-induced hepatic tumorigenesis in rat enhanced by 93[P]; intestinal-tumour induction by sc injections of azoxymethane inhibited by 139[P]; no observed effect on intragastric formation of *N*-nitrosamine from nitrite and dimethylamine 167[P]

***tert*-Butylhydroquinone,** some effect on intragastric formation of *N*-nitrosamine from nitrite and dimethylamine 167[P]

Cadmium, acetate, test for carcinogenicity by pulmonary-tumour response in mice 651; and lead, chromosomal aberrations in leucocytes of workers exposed to 158; association with hypertension in smokers and non-smokers 480; book review on: IARC monograph on carcinogenic risk from 237, toxicology and epidemiology of 65; determination of tissue levels by neutron-activation analysis 480; effects of environmental and occupational exposure to 478; inhibition of drug-metabolizing enzymes of mouse liver by 367; no renal carcinogenic or erythrogenic response to intrarenal injection in rats 370

Caffeine, absorption, tissue distribution, metabolism and excretion in man, mice, rats and squirrel monkeys 155; negative results in micronucleus test 647

Calamus oil, properties, use as fragrance raw material, status, toxicity and pharmacology of 623[M]

Calcium, acetate, test for carcinogenicity by pulmonary-tumour response in mice 651

Canada, book review on pesticide residues in Ontario 67

Caramel, 'ammonia-process': long-term feeding study in rats 523[F], short-term feeding studies in rats on two types of 509[P]

Carbamates (see also specific compounds), as insecticides, book review on chemistry, biochemistry and toxicology of 469

Carbanilide, 3,4,4′-trichloro- and 3-trifluoromethyl-4,4′-dichloro-, absorption, distribution and excretion in rats treated orally, iv or dermally with 205[P]

Carbaryl, degradation of: 1-naphthol derived from, by river-water bacteria 80, residues in green beans during heat treatment 360; *N*-nitroso-, stomach tumours in rats given oral doses of 500; with or without nitrite, no malignant tumours in dams or offspring following oral administration to rats during pregnancy 229[P]

Carbon disulphide, follow-up study on heart-disease incidence in workers exposed to 244; high acetaldehyde levels in rat and human blood following simultaneous ethanol ingestion and exposure to 244; metabolism of and effects on hepatic mixed-function oxidase system 243; tests for early diagnosis of poisoning by 244

Carcinogenesis, Ames tests on food extracts incubated with nitrite in study of gastric cancer aetiology 97[P]; apparent effect of drinking and smoking on age at onset of oral cancer in women 655; book review on: IARC monographs (Vols 10 and 11) on risks from specific chemicals and naturally occurring substances 237, IARC workshop on short-term screening tests for 66, identification of high-risk groups as an aid to cancer control 344, immunological aspects of neoplasia 470, mechanisms and causes of neoplastic change 344, tissue changes preceding malignancy 151; demonstration of possible mechanism for conveyance of environmental carcinogens to human colon as target organ 370; effects of dusts on respiratory-tumour induction by benzo[*a*]pyrene or diethylnitrosamine 499; gastric conditions and possible nitrosamine formation in patients with gastric cancer 655; mechanisms of cell transformation *in vitro* 483; possible evidence for increased cancer risk in beauticians 654; potential of micronucleus test as short-term test for 646; problems in chemical carcinogenesis and significance of environmental factors in 475; proposed method of evaluating risks of environmental carcinogens 233[R]; review of available short-term tests for 74

Carrageenan, calcium, effects of long-term oral administration on reproduction and foetal development in rats 533[P], 539[P]; *in vivo* macrophage cytotoxicity rejected as crucial factor in mechanism of immunosuppression by 251

Celery oil, liver regeneration in partially hepatectomized rats increased by 173[P]

Cetyltrimethylammonium bromide (see under Quaternary ammonium salts)

Chamomile oil, blue, liver regeneration in partially hepatectomized rats increased by ingestion of 173[P]

Chlordiazepoxide, with nitrite, induction of nervous-system tumours in rats by 269[P]

Chloroform, acute and subacute toxicity studies in rats 490; glutathione involvement in mechanisms of hepatotoxicity in rats 161

1-Chloro-2-propanol, produced by propylene oxide sterilization of chloride-containing foods, positive findings in short-term study for base-substitution mutagenicity 252

Chromium, morphological, ultrastructural and biochemical effects of chromates on ciliated cells of tracheal epithelium in the rat 254; no renal carcinogenic or erythrogenic response to intrarenal injection in rats 370; sulphate, test for carcinogenicity by pulmonary-tumour response in mice 651

Cigarette smoke (see under Tobacco)

Cinnamic acid, allyl ester, properties, use as fragrance raw material, status, toxicity and metabolism of 615[M]

Citrex S-5 (see under Sulphopolycarboxylic acids)

Citric acid, as dietary supplement, lifespan and body-weight reductions in rodents probably connected with reduced uptake of trace elements following chelation with 358; no effect on reproduction parameters in rat or mouse study 358

Citrinin, kidney lesions in mice after acute oral, sc or ip treatment with 29[P]; production by *Penicillium chrysogenum* on bread 303[P]; toxic effects in guinea-pigs following short-term gastric administration of 553[P]

Cobalt, acetate, test for carcinogenicity by pulmonary-tumour response in mice 651; and sulphide, no renal carcinogenic or erythrogenic response to intrarenal injection in rats 370

Coffee, instant: long-term feeding studies in rats on regular and decaffeinated, freeze- and spray-dried types 7[P], no tumour induction in long-term feeding studies in rats 289[P]

Colcemid, positive results in micronucleus test 647

Colchicine, positive results in micronucleus test 647
Colon, as target organ for possible environmental and dietary carcinogens, by suggested retoxication of liver metabolites 370
Copper, acetate, test for carcinogenicity by pulmonary-tumour response in mice 651
Cosmetics, announcement on US Cosmetic Ingredient Review 375; demonstration of *N*-nitrosodiethanolamine in 423[P]; possible evidence for increased cancer risk in beauticians 654
Coumarin, pharmacokinetics in man following iv administration 365
Cumene, retention in and absorption through human respiratory tract 491; urinary excretion of dimethylphenylcarbinol as index of exposure to 491
Cumin, liver regeneration in partially hepatectomized rats increased by 173[P]
Cyanide, plasma levels in patients given sodium nitroprusside infusion during surgery 490; primary effects on respiratory and cardiovascular function and secondary neuropathology in intoxication of rats by 158
Cycasin, book review on IARC monograph on carcinogenic risk from 237
Cyclamate, and cyclohexylamine metabolite, review of mutagenicity studies on 69; sodium, effect of low-fibre rat diet on toxicity of high intake of 358
Cyclohexylacetic acid, allyl ester, properties, use as fragrance raw material, status, toxicity and metabolism of 617[M]
Cyclohexylamine, as cyclamate metabolite, review of mutagenicity studies on 69
Cyclopentenone, long-term inhalation study in hamsters 491
Cyclophosphamide, positive results in micronucleus test 647
Cytology, book review on: mechanisms of intracellular and intercellular control 471, structure and function of mammalian cell membranes 642
Cytosine, 1-β-D-arabinofuranosyl-, positive results in micronucleus test 647

DDT, and derivatives, cytotoxicity and chromosomal changes induced in cultures of Chinese hamster cells by 359; book review on degradation by micro-organisms, stability in foods and feeds, removal by cooking and environmental accumulation 341; in beef, degradation during canning and cooking 359; metabolism in mouse and hamster 157
Dehydroretronecine, as monocrotaline metabolite, inhibition of yeast alcohol dehydrogenase *in vitro* by 419[P]
Detergents (see Surface-active agents or specific compounds)
Dextran blue, absorption from intestine of piglets 0–6 days after birth 87
Diaminotoluene (see Toluenediamine)
Dibenzo-*p*-dioxin, 2,3,7,8-tetrachloro-: direct and delayed effects of human exposure to 481, enzyme-inducing activity in hens' eggs, rats and mice 482, *in vitro* studies of effects on mitosis and nucleic acid synthesis 481, morphological changes in monkeys after 9-month dietary administration of low levels of 401[P], single-dose oral toxicity studies in rats and mice 481, tissue distribution, retention and excretion in orally dosed rats 482
***trans*-1,4-Dichlorobutene-2,** few local and no systemic tumours induced by sc or ip injection or skin application in mouse carcinogenicity study 246
Dichloroethylene, (1,1-, see Vinylidene chloride), *cis-* and *trans*-1,2-, no mutagenic activity in bacterial tests before or after metabolic epoxidation 85
Dichloromethane, effects on guinea-pig liver of acute or subacute inhalation with or without ethanol 652
Dieldrin, re-evaluation of tumour data from earlier 2-yr feeding study in rats 360; reproduction study in mice 252
Diethylamine, occurrence in foods, stimulants and surface waters 275[P]
Diethylenetriaminepentaacetic acid, foetotoxicity of sc-administered calcium but not zinc salt in mouse reproduction study and possible levels of toxicity to human foetus 362
Diisocyanate, toluene: apparent inhalational allergic pneumonitis in workers exposed to 255, moderation of β-adrenergic function involved in production of pulmonary damage by 84, possible immunological basis of pulmonary disease due to 365, respiratory and neurological effects in fireman exposed to 493
Dimethylamine, occurrence in foods, stimulants and surface waters 275[P]
Dimethyldodecylamine, with nitrite, induction of urinary-bladder tumours in rats 269[P]
Dimethylnitramine, hepatic, renal and other tumours induced in mice by 499
Dimethylphenylcarbinol, as main urinary metabolite of and index of exposure to cumene 491
Dioctyl sulphosuccinate, toxicity in horses and guinea-pigs 159
Diquat, book review on: environmental fate and toxicity of 641, inactivation in soils 67
Dodecylisoquinolinium bromide, corneal irritancy tests on Isothan Q-15 in nine species 131[P]
Drugs, book review on: adverse effects elicited by 241, comprehensive survey of pharmacology of therapeutic compounds 241, human microsomal-enzyme induction and specific metabolic disturbance by anticonvulsants 470, pharmacokinetics of 152, teratogenic effects of 152

Emulsifier YN, long-term feeding study in mice 1[P]
Endothall, book review on environmental fate and toxicity of 641
Environment, book review on occurrence and fate of chemical contaminants in 469
Enzymes, alkaline phosphatase (predominantly hepatic), but not microsomal enzymes, induced in beagles by low doses of an ethanol–propylene glycol–chloroform combination 135[P]; book review on value of enzymology in diagnosis of genetic disorders, prenatal diseases and other clinical conditions 472; interspecies comparison of drug-metabolizing capacity 87, 497; microsomal, inhibited in mouse liver by cadmium chloride treatment 367; proteolytic: asthmatic reactions after occupational inhalation of papain dust 261, inhalation and histamine-release studies of subtilisin-containing preparation in guinea-pigs 261
Epidemiology, book review on basic methods and difficulties in 471
Erucic acid, in rapeseed oil, effects in rats, ducklings, chicks and pigs 350; possible biochemical mechanisms underlying adverse effects in animals and man 350; sex difference in susceptibility of rats to cardiopathogenicity of 349
Essential oils (see also specific materials), and components, effect of sc or oral administration on liver regeneration in partially hepatectomized rats 173[P]
Estragole, and related compounds, review of metabolism and toxicity of 645
Ethanol, acetaldehyde levels in rat foetus and placenta during oxidative metabolism of 77; effects of: alcoholic congeners on elimination of, by isolated perfused rat liver 653, inadequate diets on hepatic responses of rats to alcoholic beverages 259; no detectable effect on tin absorption in rats dosed with stannous chloride 147[P]; placental transport in ewes and effect on foetal brain and acid-base balance 78
Ethoxysulphates (see under Polyoxyethylene condensates)

Ethylenebisdithiocarbamate, comparative studies on derivatives related to ethylene thiourea suggesting some link between molecular structure and teratogenic effect 361

Ethylene thiourea, and related compounds, comparative teratogenicity studies in rats 361; apparently responsible for observed teratogenicity in rats, rather than metabolite 80; distribution, metabolism and excretion in pregnant rats 80; epidemiological study of possible foetal malformations and thyroid cancer following industrial exposure to 363; foetal-malformation patterns in relation to day of administration of single oral dose to pregnant rats 157; long-term feeding studies in rats and hamsters 649

Ethylidene gyromitrin, as toxic component of false morels, acute oral toxicity in rabbits, rats and chickens 575[P]

Ethylurea, with methylmercury and nitrite, synergistic effects on reproduction and offspring survival in rats 283[P]

Eye, interspecies comparison of corneal irritancy of test shampoos and detergents 131[P]

False morel, acute oral toxicity studies on ethylidene gyromitrin present in 575[P]

FAO/WHO, report on food antioxidants, solvents and other additives (20th Report) 639

Fast Red E, reduction by whole-cell extracts of contents of rat caecum 487

Fatty acids (see also specific compounds), chlorinated, comparative metabolism *in vitro* by various adult and foetal rat tissues 331[P]; ratio of unsaturated to saturated, suggested as possible factor influencing cardiopathogenicity of rapeseed oils 349

FEMA, review of criteria used for GRAS evaluation by 457[R]

Fenitrothion, book review on formulations, analysis, properties and uses of 341

Fennel oil, liver regeneration in partially hepatectomized rats increased by 173[P]

Fibre, dietary, increase in toxicity of individual or combinations of food additives ingested in low-fibre diet by young rats 358; synthetic, studies of respiratory disease in textile workers and guinea-pigs exposed to 86

Firemaster BP-6 (see under Polybrominated biphenyls)

Flavourings (see also specific compounds), criteria used by FEMA for GRAS evaluation of 457[R]; FAO/WHO discussion on priorities for evaluation of 639

Flour, chlorinated lipid and gluten fractions, absorption, tissue retention and excretion studies on water-soluble extracts in rats 105[P]

Fluorocarbons (see also Polytetrafluoroethylene), alleged interaction with ozone layer in upper atmosphere 74; inhalation studies in rats, dogs, hamsters, rabbits and man 72; metabolism, distribution, biological half-lives and release of fluoride *in vivo* from 73

5-Fluorouracil, positive results in micronucleus test 647

Food, Ames mutagenicity tests on extracts of fish, meat, cereal and vegetable samples after incubation with nitrite 97[P]; book review on toxic food contaminants 65

Food additives, book review on control and safety of food chemicals 641

Formaldehyde, as probable factor in respiratory-tract reactions to occupational exposure to phenol–formaldehyde resins 84

Fragrance materials (see also specific components), phototoxicity testing of components of 55[P], 265[L]

Fungal toxins, produced by subspecies of *Aspergillus ochraceus*, subacute toxic effects in mice 411[P]

Furfuryl mercaptan, short-term oral toxicity study in rats 383[P]

Furylfuramide, DNA damage in bone-marrow chromosomes from rats treated ip or orally with 78

Gallic acid, propyl ester: inhibition of nitrosopyrrolidine formation in frying bacon by 79, some effect on intragastric formation of *N*-nitrosamine from nitrite and dimethylamine 167[P]

Garlic, small epidemiological study on postulated influence on factors involved in atherosclerosis 493

Glutamic acid, monosodium salt: reproductive-organ development in young rats, mice and hamsters treated with 348, review of effects on brain tissue in various species 347

Glutaraldehyde, book review on chemistry and biological applications of 472

Goitrin, D- and L-, no teratogenic effect demonstrated in rat study 61[P]

Gold, no renal carcinogenic or erythrogenic response to intrarenal injection in rats 370

Gossypol, book review on metabolism and physiological and nutritional effects in various species 341

Guaiene, liver regeneration in partially hepatectomized rats increased by ingestion of 173[P]

Hair dyes (see also specific constituents), lifetime percutaneous studies in mice and rabbits 601[P]; no toxic effects in adult rabbits or interference with reproduction in rats in skin-painting studies of 12 composite formulations 366; skin- and eye-irritancy studies on 12 constituents in rabbits and acute oral toxicity in rats 607[P]

Hair shampoos, corneal irritancy tests on commercial formulations in nine species 131[P]; demonstration of *N*-nitrosodiethanolamine in 423[P]

Helenalin, as constituent of range plants, negative result in Ames mutagenicity test 225[P]

Heptane, and/or other low-boiling paraffins as probable cause of shoemakers' neuropathy 258

Heptanoic acid, allyl ester, properties, use as fragrance raw material, status, toxicity and metabolism of 619[M]

Herbicides (see also specific compounds), book review on: bioassay techniques for types formulated for foliar application 341, metabolic fate in experimental and farm animals 341, monographs relating to toxicology of 641

Hexachlorobenzene, influence of dosage vehicle on absorption and tissue distribution in rats 80; ultrastructural changes induced in rat liver by 80

Hexachlorophene, biochemical studies of brain oedema produced in rats by 363; contamination of surface waters from industrial and other use of 353; effects of vitamin-A treatment of rats on toxicity of 35[P]; neurotoxicity in rats, pigs and cats, and suggested alteration of cellular membrane 353; percutaneous absorption studies 353; three-generation feeding and teratology studies 354

Hexane, and/or other low-boiling paraffins as probable cause of shoemakers' neuropathy 258; *n*-, effects on central and peripheral nervous systems in rats and printing workers 492

Hexanetriol, sensitivity to, demonstrated in cases of contact dermatitis due to a skin ointment 84

Histology, book review on: an introduction to human histopathology 240, techniques used in 643

4-Hydroxy-3-methoxybenzaldehyde (see Vanillin)

Hymenovin, as toxic constituent of Western bitterweed, positive results in Ames mutagenicity test 225[P]

Hyperkinesis, apparent support for Feingold hypothesis deemed experimentally unsound 357

Immunology, book review on: molecular and cellular basis of immunity 240, tolerance as an integral part of the immune response 240

Industrial hazards, book review on: early detection of health impairment due to 63, toxicity of specific chemicals 642

Insecticides (see also specific compounds and groups), for use in stored cereal grains, book review on metabolism and terminal residues of 341

Intestine, absorption of dextran blue from, in newborn and young pigs 87; of neonatal pigs and mature rats, persorption of macromolecules in different regions of 368
Iodine, suggested implication of low dietary levels in high incidences of breast, endometrial and ovarian cancer 162
Iron, effect of: acute ferrous sulphate poisoning on rat gut 361, intratracheal instillation of ferric oxide dust on respiratory-tumour induction by benzo[*a*]pyrene or diethylnitrosamine 499; 2,4-pentanedione, test for carcinogenicity by pulmonary tumour response in mice 651
Isoamyl alcohol, as congener in alcoholic drinks, effect on ethanol elimination by isolated perfused rat liver 653
***p*-Isopropylbenzaldehyde,** liver regeneration in partially hepatectomized rats increased by 173[P]
Isopropylbenzene (see Cumene)
Isosafrole, liver regeneration in partially hepatectomized rats increased by 173[P]

Kidney, book review on use of tubule cells in metabolic studies 342

Laboratory animals, comparative activities of drug-metabolizing enzymes in various species 497
Lead, as co-contaminant in cadmium-containing atmosphere associated with chromosomal aberrations in leucocytes of exposed workers 158; book review on toxicology and epidemiology of 65; effect of: dietary level and food restriction on retention of, in rats 489, subtoxic levels on pregnant women and levels in foetal, maternal and placental blood 488; from lead-soldered electric kettles, cases of poisoning by 650; no renal carcinogenic or erythrogenic response to intrarenal injection in rats 370; subacetate, test for carcinogenicity by pulmonary-tumour response in mice 651
Limonene, *d*-: species differences in metabolism of 251, urinary metabolites and postulated metabolic pathways in rats 77
Linoleic acid, and triglyceride, absorption from rat gut reduced by chlorination 101[P]; chlorinated: mammary transfer in rats 183[P], rate of metabolism by rat tissues *in vitro* 331[P]
Linolenic acid, and triglyceride, absorption from rat gut reduced by chlorination 101[P]; chlorinated, mammary transfer in rats 183[P]
Liver, book review on: recent progress in study of liver diseases 342, use of isolated cells in metabolic studies 342; microsomal enzymes in beagles not raised in parallel with alkaline phosphatase in serum and liver following ethanol–propylene glycol–chloroform treatment 135[P]
Locoweed, effects on rats and evidence for urinary excretion of unchanged toxin by sheep following ingestion of 366
Lucanthone, liver-tumour induction in rats treated with 269[P]
Lysinoalanine, renal lesions in rats following dietary administration of 117[P]; synthesis and physico-chemical properties 109[P]
Lysosomes, book review on: normal and pathological functions of 344, recent studies on biological and pathological aspects of 343

Mace, liver regeneration in partially hepatectomized rats increased by 173[P]
Manganese, sulphate, test for carcinogenicity by pulmonary-tumour response in mice 651
Matricaria, flowers, liver regeneration in partially hepatectomized rats increased by ingestion of 173[P]
Medical plastics (see specific polymers or under Plastics)
6-Mercaptopurine, positive results in micronucleus test 647
Mercury, acute effects of mercuric chloride on cultures of mouse-leukaemia cells 81; book review on toxicology and epidemiology of 65; methyl-: synergistic effects with nitrite and ethylurea on reproduction and offspring survival in rats 283[P], variations in accumulation and tissue distribution in guinea-pigs, cats, rats and man and in animals, fish and birds used as food 70; UK report on environmental occurrence and fate, sources and effects of human exposure 467
Metabolism, book review on use of liver cells and kidney tubules in studies of 342; of drugs, interspecies comparison of efficacy of lung, liver and kidney enzymes in 87; of foreign compounds in mammals, book review on 239
Metals (see also specific elements and their compounds), book review on toxic effects, epidemiology and dose-response relationships 65
Methacrylate, methyl, effects of subacute inhalation of vapour by rats 257; polymer cements, direct effects on isolated heart muscle 83
Methanesulphonic acid, methyl and ethyl esters, positive results in micronucleus test 647
Methanol, different metabolic patterns in rat and monkey and possible use of folate-deficient rat for toxicity studies relevant to man 256
Methapyrilene, with nitrite, induction of liver tumours in rats 269[P]
Methotrexate, as treatment for psoriasis, follow-up of patients indicating hepatotoxicity rather than carcinogenicity 159; positive results in micronucleus test 647
***N*-Methylaniline,** occurrence in foods, stimulants and surface waters 275[P]
***N*-Methylbenzylamine,** occurrence in foods, stimulants and surface waters 275[P]
Methyl-1-(butylcarbamoyl)-2-benzimidazol-2-yl carbamate (see Benomyl)
Methyl butyl ketone, *n*-butyl isomer: alone or with methyl ethyl ketone, blood levels and toxic effects in inhalation study in rats 256, axonal degeneration induced in central nervous system of rats by 492, further evidence for role in peripheral neuropathy from paint-sprayer survey 159, response of feline nervous system to repeated intoxication by 492, urinary metabolites in rats after ip injection of 256; isobutyl isomer, response of feline nervous system to repeated intoxication by 492
Methylchloroform (see 1,1,1-Trichloroethane)
3-Methylcholanthrene, and dimethylnitrosamine, synergistic effect of simultaneous administration on pulmonary-tumour induction in rats and mice 369
Methylene dichloride (see Dichloromethane)
Methyl ethers, bischloro- and chloromethyl, tumour incidences in epidemiological and animal studies 244; carcinogenicity studies (skin application and sc or ip injection) in mice on bis-1,2-(chloromethoxy)ethane, bis-1,4-(chloromethoxy)butane, bis-1,6-(chloromethoxy)hexane, bis-1,4-(chloromethoxy)-*p*-xylene and tris-1,2,3-(chloromethoxy)propane 246
Methyl ethyl ketone, properties, use as fragrance raw material, status, toxicity, metabolism and pharmacology of 627[M]; response of feline nervous system to repeated intoxication by 492; with methyl *n*-butyl ketone, blood levels and toxic effects following simultaneous exposure of rats to 256
***N*-Methylphenethylamine,** occurrence in foods, stimulants and surface waters 275[P]
Methyl protocatechuic aldehyde (see Vanillin)
Mitomycin C, positive results in micronucleus test 647
Molybdenum, trioxide, test for carcinogenicity by pulmonary-tumour response in mice 651
Moniliformin, produced by *Fusarium moniliforme* from maize, toxicity in ducklings and rats 579[P]
Monosodium glutamate (see under Glutamic acid)
Mushrooms, short-term oral toxicity of *Paxillus involutus* to rats 445[P]

Mutagenesis, Ames tests on food extracts after incubation with nitrite 97[P]; concentration of mutagens in body tissues of mussels and possible use in monitoring marine environments for contamination 497; review of advantages and disadvantages of micronucleus test for 646

Naphthionic acid, as amaranth metabolite, serum levels and excretion after administration to rats by gavage or after amaranth administration by gavage or in diet or drinking-water 77

1-Naphthol, as major hydroxylation product of carbaryl, degradation by bacteria from river water 80; skin and eye irritancy in rabbits and acute oral toxicity in rats 607[P]

2-Naphthylamine-1-sulphonic acid, or metabolites, excretion following oral or iv administration to rats 487

α-Naphthylisothiocyanate, mechanism of hepatotoxicity in mice and rats 484

Neutronyx 600, aromatic polyglycol ester condensate, corneal irritancy tests in nine species 131[P]

Nickel, acetate, test for carcinogenicity by pulmonary-tumour response in mice 651; and sulphur-containing derivatives, variations in sarcoma-inducing potential of im injections of suspensions of 370; book review on IARC monograph on carcinogenic risk from 237; diffusion through and interactions with epidermis as factor in hypersensitivity responses 83; metal and its monosulphide, no renal carcinogenic or erythrogenic response to intrarenal injection in rats 370; methods of testing for sensitization in guinea-pigs 489; nasal-mucosa histology of workers exposed to 362; oxide, effects of inhalation on hamster lungs, body weight and survival with or without exposure to cigarette smoke 82; subsulphide, renal tumour induction and high erythrocyte levels in rats given intrarenal injection of 370

Nitrate, no correlation between residual levels in Chinese foods and nitrosamine content 143[P]; potassium, no detectable effect on tin absorption in rats dosed with stannous chloride 147[P]

Nitrilotriacetic acid, negative mutagenicity study in Drosophila 496

Nitrite, no correlation between residual levels in Chinese foods and nitrosamine content 143[P]; sodium, synergistic effects with methylmercury and ethylurea on reproduction and offspring survival in rats 283[P]

Nitrobenzene, methaemoglobin formation in rats resulting from reduction of, by gut bacteria 262

Nitroprusside, sodium, plasma cyanide and thiocyanate in surgical patients infused with 490

Nitrosamine, diethyl-: and dimethyl-, presence in deionized-water samples demonstrated by GLC 441[P], effect of intratracheal instillation of various dusts on respiratory-tumour induction by 499, effect of tryptophan supplement on liver-tumour incidence but not on oesophageal tumours in rats fed diet containing 431[P], effects in hamsters exposed to tobacco smoke and given sc injections of 369; dimethyl-: and 3-methylcholanthrene, synergistic effect of simultaneous administration on pulmonary-tumour induction in rats and mice 369, and *N*-nitrosopyrrolidine, levels in Chinese food products 143[P], inhibition of metabolism possibly responsible for suppression of mutagenic potential by other nitrosamines including nitrososarcosine 251, intragastric formation little affected by edible antioxidants 167[P], no evidence on mechanism of carcinogenicity derived from dimethylnitramine study in mice 499, no suppression of acute toxicity associated with DMN-demethylase inhibition by other nitrosamines 252; effect of route and dosage schedule on tumour induction by nitrosoheptamethyleneimine in rats 88; estimation of relative degrees of risk from different nitroso compounds present in foods 233[R]; formation from ingested nitrite and environmental amines tested in rats by tumour-induction study 269[P]; gastric pH, bacteria and nitrite levels in patients with and without gastric cancer, in relation to *in vivo* formation of 655; *N*-nitrosodiethanolamine detected in cosmetics, lotions and shampoos 423[P]; nitrosopyrrolidine formation during bacon frying inhibited by propyl gallate, ascorbyl palmitate, sodium ascorbate or piperazine hydrate 79; volatile compounds determined in deionized water by GLC–MS 437[P]

***N*-Nitrosocarbaryl** (see under Carbaryl)

***N*-Nitrosoguanidine,** *N*-methyl-*N′*-nitro-, negative results in micronucleus test 647

Nucleosides, *C*-, book review on chemistry and biochemistry of 472

Nutmeg oil, liver regeneration in partially hepatectomized rats increased by 173[P]

Nylon, fibre, lung lesions in guinea-pigs exposed to high dust concentrations of 86

Ochratoxin A, alone and with rubratoxin B, acute oral effects in infant rats 23[P]; distribution and excretion after ip administration to rats 199[P]; occurrence in feedstuffs 246; production by subspecies of *Aspergillus ochraceus* and subacute effects in mice 411[P]; renal changes induced in rats, pigs, dogs and chick and possible relevance to human endemic nephropathy 246; toxic effects in guinea-pigs following short-term gastric administration of 563[P]

Oleic acid, absorption from rat gut reduced by chlorination 101[P], 105[P]; and dichlorinated derivative, *in vitro* metabolism by adult and foetal rat tissues 331[P]; placental and mammary transfer in rats compared with that of chlorinated derivative 183[P]

Onions, small epidemiological study on postulated influence on factors involved in atherosclerosis 493

Optical brighteners (see also specific compounds), negative results in Ames tests before and after exposure to rat-liver preparation 262

Orange G, 1-yr feeding study in ferrets 379[P]

Organophosphorus pesticides (see also specific compounds), book review on re-entry safety after application of 641

Organotin compounds (see under Tin)

Paraquat, book review on inactivation in soils 67

Parsley oil, liver regeneration in partially hepatectomized rats increased by 173[P]

Patulin, and cysteine adducts, embryotoxicity and teratogenicity demonstrated in hens' eggs 495

Penicillic acid, production by subspecies of *Aspergillus ochraceus* and subacute effects in mice 411[P]

Pentachlorophenol, inhalation studies in rats 650

Pentane, and/or other low-boiling paraffins as probable cause of shoemakers' neuropathy 258

Perchloroethylene (see Tetrachloroethylene)

Persorption, of dextran blue from intestine of piglets 0–6 days after birth 87; of macromolecules in different intestinal regions of neonatal pigs and of normal and protein-deficient mature rats 368

Pesticides (see also specific compounds and groups), book review on: automated routine analytical procedures 67, secondary effects on complex ecological systems 67

Pharmacology, book review on release of active compounds in parasitic diseases 472

Pheneturide, and other anticonvulsant therapy, effects on calcium and vitamin D metabolism and mineral content of bone 247

Phenobarbitone, 2-acetylaminofluorene-induced hepatic tumorigenesis in rat enhanced by 93[P]; and other anticonvulsant therapy, effects on calcium and vitamin D metabolism and mineral content of bone 247

Phenol-formaldehyde resin, respiratory-tract effects in exposed workers 84

Phenols, brominated, as principal metabolites of mono-, di- and tribromobenzenes in rabbits 255
Phenylacetic acid, allyl ester, properties, use as fragrance raw material, status, toxicity and metabolism of 621[M]
Phenylenediamine, *p*-: lifetime skin application studies in mice and rabbits 601[P], skin and eye irritancy in rabbits and acute oral toxicity in rats 607[P]; 4-methoxy-*m*-, skin and eye irritancy in rabbits and acute oral toxicity in rats 607[P]; 4-nitro-*o*- and 2-nitro-*p*-, skin and eye irritancy in rabbits and acute oral toxicity in rats 607[P]
Phenytoin, and other anticonvulsant therapy, effects on calcium and vitamin D metabolism and mineral content of bone 247
Phosphate, triaryl, biochemical and behavioural effects in fish exposed to lubricating oil consisting of 85; tri-*o*-cresyl, unlikely to be major cause of Italian shoemakers' neuropathy 258
Phthalic acid, di-(2-ethylhexyl) ester: distribution and biliary metabolites in trout exposed to water containing 253, short-term feeding study in rats 389[P], transfer to patients during haemodialysis 253; hydrolysis of dimethyl, diethyl, di-*n*-butyl, dicyclohexyl, di-*n*-octyl and di-(2-ethylhexyl) esters by rat-gut contents 17[P]
Piperazine, hydrate, inhibition of nitrosopyrrolidine formation in frying bacon by 79
Piperidine, occurrence in foods, stimulants and surface waters 275[P]
Piperonyl butoxide, teratogenicity study in rats 337[P]
Plastics, medical, influence of implant shape on degree of tissue reaction to 498
Poly-AO, polymeric phenols as potential antioxidants for foods 357
Polybrominated biphenyls, tissue and milk levels: in exposed humans 157, in ruminants, following ingestion of 157; weak teratogenicity of Firemaster BP-6 fed to pregnant rodents 257
Polychlorinated biphenyls, book review on residues in US silos 67; excretion and metabolism of pentachloro- compound in squirrel monkey 335[P]; identified in tissues of 'Yusho' patients and others 195[P]
Polychlorinated dibenzofurans, penta- and hexachloro-, identified in tissues of 'Yusho' patients 195[P]
Polyethylene, sample shape as factor in tissue reactions to implants of 498
Polyethylene glycol 1500, no sensitization reactions in group of patients with contact dermatitis due to a skin ointment 84
Polyoxyethylene condensates, alkyl ethoxysulphates, effect on mouse foetuses of dermal or sc administration of detergent formulation containing 367; alkyl polyethoxylates: acute oral, ip, dermal, ocular and inhalation effects in man and/or other species 309[P], effect on mouse foetuses of dermal or sc administration of detergent formulation containing 367, subacute tests for oral (rats) and dermal (rabbits) toxicity and for human contact-sensitization 319[P]
Polypropylene, sample shape as factor in tissue reactions to implants of 498
Polytetrafluoroethylene, reaction of rat lung to particulate components of pyrolysis products of 491; sample shape as factor in tissue reactions to teflon implants 498
Polyvinyl chloride, sample shape as factor in tissue reactions to implants of 498
Ponceau 4R, reduction by whole-cell extracts of contents of rat caecum 487
Ponceau 6R, reduction by whole-cell extracts of contents of rat caecum 487
Primidone, and other anticonvulsant therapy, effects on calcium and vitamin D metabolism and mineral content of bone 247
***n*-Propanol,** as congener in alcoholic drinks, effect on ethanol elimination by isolated perfused rat liver 653
Propylene glycol, sensitivity to, demonstrated in cases of contact dermatitis due to a skin ointment 84
Propylene oxide, as sterilant for foods, positive findings in short-term mutagenicity studies on 1-chloro-2-propanol from 252
Protocatechualdehyde-3-methylether (see Vanillin)
Pyrithione, sodium and zinc, absorption, retention and excretion following oral or ip administration to rats, rabbits and monkeys 49[P]; zinc: absorption, distribution and excretion following oral or dermal administration to rabbits 496, neurological evaluation of generalized weakness in rats treated with 43[P]
Pyrrolidine, occurrence in foods, stimulants and surface waters 275[P]

Quaternary ammonium compounds, cetyltrimethylammonium bromide, subacute toxicity in rats 160; corneal irritancy tests on an alkyldimethylbenzylammonium chloride in nine species 131[P]

Rapeseed, oil: components other than erucic acid as possible factors in adverse effects of 349, review of toxic effects of erucic acid and/or other components of 348; protein: or flour, feeding studies in rats, mice and dogs 494, teratogenicity studies in rats and mice 494
Resorcinol, studies on skin and eye irritancy in rabbits and acute oral toxicity in rats 607[P]
Rubratoxin B, alone and with aflatoxin B_1 or ochratoxin A, acute oral effects in infant rats 23[P]

Safrole, and related compounds, review of metabolism and toxicity of 645; book review on IARC monograph on carcinogenic risk from 237; liver regeneration in partially hepatectomized rats increased by 173[P]
Salicylanilide, halogenated derivatives, primary sensitization and cross-sensitivity in guinea-pigs 325[P]
Sassafras, liver regeneration in partially hepatectomized rats increased by 173[P]
Selenium, book review on environmental significance of 641
Sesquiterpene lactones, varying results in Ames mutagenicity tests 225[P]
Silicone rubber, sample shape as factor in tissue reactions to implants of 498
Silicones, dimethylpolysiloxanes, environmental studies indicating persistence, low toxicity and immobilization by particulate binding in aqueous systems 79
Skin, of mouse, man and swine, phototoxicity testing of fragrance raw materials on 55[P]
Smoking (see under Tobacco)
Solasodine, solanum alkaloid present in egg plant, foetal malformations and high level of resorptions in hamsters following exposure to 260
Solvents (see also specific compounds), for food, FAO/WHO toxicological evaluation 639
Soy sauce, gastric lessions but no tumour induction in long-term feeding study of 'shoyu' in rats 495
Starch, suggested involvement of talc in granulomatous reactions to surgical-glove powders containing 87
Statistics, book review on pocket calculators and their use in 242
Stearyl alcohol, sensitivity to, demonstrated in cases of contact dermatitis due to a skin ointment 84
Sterigmatocystin, book review on IARC monograph on carcinogenic risk from 237; occurrence in breads, optimal conditions of production 260
2-(4-Styryl-2-sulphophenyl)-2*H*-naphtho[1,2-*d*]triazole, sodium salt, negative results in Ames tests 262
Sucrose, esters and sucroglycerides, FAO/WHO toxicological evaluation 639; no detectable effect on tin absorption in rats dosed with stannous chloride 147[P]
Sulphopolycarboxylic acids, sodium salts, negative mutagenicity study on Citrex S-5 in Drosophila 496

Sunflower oil, emulsion, no detectable effect on tin absorption in rats dosed with stannous chloride 147^P
Surface-active agents (see also specific compounds), acute oral and parenteral studies on detergent formulations containing alkyl polyethoxylates 309^P; corneal irritancy tests on detergent products in nine species 131^P; no conclusive evidence of embryotoxic or teratogenic effects in mice treated sc or dermally with linear alkylbenzene sulphonate or polyoxyethylene alkyl ether/alkyl ethoxysulphate formulations 367; subacute dermal studies in rabbits and usage tests in man on detergent formulations containing alkyl polyethoxylates 319^P

2,4,5-T (see 2,4,5-Trichlorophenoxyacetic acid)
Talc, lung deposition of particles following inhalation by hamsters 121^P; neutron-activated, studies on leaching of radionuclides into serum or acid media 589^P; pulmonary deposition, translocation and clearance in hamsters following inhalation of 213^P; study of baby-powder inhalation by hamsters 121^P; suggested involvement in granulomatous reactions generally attributed to starch in surgical-glove powders 87; very low absorption from gut following ingestion by hamsters 453^P
Tarragon oil, liver regeneration in partially hepatectomized rats increased by 173^P
Teflon (see under Polytetrafluoroethylene)
Tenulin, as constituent of range plants, negative result in Ames mutagenicity test 225^P
Teratogenesis, book review on effect of drugs and other chemicals on foetal development 152
Tetrachloroethylene, experimental human studies suggesting limited use of respiratory elimination measurements to provide index of exposure 160; no mutagenic activity in bacterial tests before or after metabolic epoxidation 85
Thiocyanate, plasma levels in patients given sodium nitroprusside infusion during surgery 490
Thioglycollic acid, sodium salt, lifetime skin application studies in mice and rabbits 601^P
Thiophosphoramide, triethylene, positive results in micronucleus test 647
Thio-TEPA (see Thiophosphoramide, triethylene)
Thiourea, and derivatives, comparative teratogenicity studies in mice 361
Tin, chloride, test for carcinogenicity by pulmonary-tumour response in mice 651; di-*n*-octyl-, thymic atrophy induced in rats by 81; inorganic, no effect of dietary components on absorption and tissue distribution demonstrated in rats intubated with stannous chloride 147^P; triethyl-: binding to myelin isolated from rat brain 258, biochemical studies of brain oedema produced in rats by 363, electrophysiological, morphological and biochemical studies of neuropathy induced in rats by 258
Tobacco, apparent effect of smoking and drinking on age at onset of oral cancer in women 655; effects of smoke exposure combined with diethylnitrosamine injections in hamsters 369; influence of cigarette-smoke exposure on effects of inhalation of nickel oxide by hamsters 82
Tobias acid (see 2-Naphthylamine-1-sulphonic acid)
Toluenediamine, 2,4-, predominantly phenolic metabolites identified in rabbits, rats and guinea-pigs 261; 2,5-: in formulations of hair-dye type, percutaneous absorption studies in rats and dogs 595^P, skin and eye irritancy in rabbits and acute oral toxicity in rats 607^P; teratogenicity study in mice, involving single sc dose during pregnancy 447^P
Toxaphene, reproduction studies in rat and mouse, species differences in foetotoxicity 360
Toxicity testing (see also under Carcinogenesis, Eye, Mutagenesis and Skin), book review on human experimentation 151; comparison of activities of drug-metabolizing enzymes of rhesus monkey with those of other species 497
Toxicology, book review on: importance of risk–benefit analysis in practical application of safety assessments 66, systematic survey of effects of industrial chemicals 642; importance of bioactivation and detoxication mechanisms in 355
Trenimon, positive results in micronucleus test 647
Triazines, substituted, book review on chemistry of 472
Trichloroethane, 1,1,1-: book review on assessment of safety as environmental chemical 239, effects of dosage route and vehicle on liver-enzyme activity and metabolism of, in mice 364
Trichloroethylene, behavioural studies in rats repeatedly exposed to 85; book review on assessment of safety as environmental chemical 239; no mutagenic activity in bacterial tests before, but some after, metabolic epoxidation 85
2,4,5-Trichlorophenoxyacetic acid, excretion following daily administration to rats and effect on renal organic-ion transport 297^P
Triethylenemelamine, positive results in micronucleus test 647
2,3,5-Tri(ethylenimino)-*p*-benzoquinone (see Trenimon)
Triolein, absorption from rat gut reduced by chlorination 101^P
Tris-(2,3-dibromopropyl) phosphate, migration from textiles, and percutaneous absorption and excretion of urinary metabolite in rat and man 257
Tryptophan, effect of dietary supplement on liver-tumour incidence in rats also fed diethylnitrosamine or 3′-methyl-4-*N*-dimethylaminoazobenzene 431^P

United Kingdom, report of: Chief Inspector of Factories 63, Laboratory of the Government Chemist 237

Vanadium, 2,4-pentanedione, test for carcinogenicity by pulmonary-tumour response in mice 651
Vanillin, properties, use as fragrance raw material, status, toxicity, metabolism and pharmacology of 633^M
Vetiverol, and vetivert oil, liver regeneration in partially hepatectomized rats increased by ingestion of 173^P
Vincristine, positive results in micronucleus test 647
Vinyl chloride, determination in PVC-packed foods by headspace gas chromatography 547^P; no mutagenic activity in bacterial tests before, but some after, metabolic epoxidation 85
Vinylidene chloride, no mutagenic activity in bacterial tests before, but some after, metabolic epoxidation 85
5-Vinyl-2-oxazolidinethione (L-(−)-, see Goitrin)
Vitamin A, effect on hexachlorophene toxicity in rats 35^P

Warfarin, evidence suggesting teratogenic effects following maternal anticoagulant treatment with 162
WHO (see also FAO/WHO), report on early detection of health impairment due to industrial hazards 63
Wool, dusts, effects of inhalation on respiratory function in man 653

Zearalenone, osteogenic effect demonstrated in teratogenicity study in rats 495
Zinc, acetate, test for carcinogenicity by pulmonary-tumour response in mice 651

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