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CONTENTS

Page

RESEARCH SECTION

Effects of chronic oral administration of erythrosine in the mongolian gerbil (*T. F. X. Collins and E. L. Long*) 233

Short-term toxicity of hydratropic aldehyde in rats (*D. Pelling, I. F. Gaunt, K. R. Butterworth, J. Hardy, A. B. G. Lansdown and S. D. Gangolli*) 249

Long-term toxicity of cyclohexylamine hydrochloride in the rat (*I. F. Gaunt, J. Hardy, P. Grasso, S. D. Gangolli and K. R. Butterworth*) 255

Long-term toxicity of cyclohexylamine hydrochloride in mice (*J. Hardy, I. F. Gaunt, J. Hooson, R. J. Hendy and K. R. Butterworth*) 269

Effects of butylated hydroxyanisole on *Tetrahymena pyriformis* (*J. G. Surak, R. L. Bradley, Jr., A. L. Branen and E. Shrago*) 277

A comparison of the distribution and elimination of oleic and chlorinated oleic acids and their metabolites in rats (*H. M. Cunningham and G. A. Lawrence*) 283

Frequency of polyploid cells in the bone marrow of rats fed irradiated wheat (*K. P. George, R. C. Chaubey, K. Sundaram and A. R. Gopal-Ayengar*) 289

Aflatoxin production on irradiated foods (*E. Priyadarshini and P. G. Tulpule*) 293

Teneur minimale du régime en lindane induisant les monoxygenases microsomales chez le rat (*M. A. Pélissier et R. Albrecht*) 297

Phototoxicity and photocarcinogenesis: Comparative effects of anthracene and 8-methoxypsoralen in the skin of mice (*P. D. Forbes, R. E. Davies and F. Urbach*) 303

MONOGRAPHS

Monographs on fragrance raw materials (*D. L. J. Opdyke*) 307

Continued on inside back cover

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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

Vinyl chloride—Part I (p. 347); The metabolic fate of polychlorinated biphenyls (p. 349); More light on hexachlorobenzene (p. 351); Ultra-short-term tests in bacterial systems (p. 353).

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

FLAVOURINGS, SOLVENTS AND SWEETENERS: Odorous metabolism (p. 355); Maternal alcoholism and its effects on pregnancy (p. 355)—MISCELLANEOUS DIRECT ADDITIVES: Iodine hazard for the goitrous? (p. 356)—AGRICULTURAL CHEMICALS: Pesticide residue levels for German babies (p. 356)—PROCESSING AND PACKAGING CONTAMINANTS: Alkane metabolism in rats (p. 357); Further steps in DEHP metabolism (p. 358)—THE CHEMICAL ENVIRONMENT: Arsenic in the air (p. 358); Searching for the basis of chromium carcinogenicity (p. 358); Methylation of lead by micro-organisms (p. 359); Lead intake from printed paper (p. 359); Lead strikes the foetus (p. 360); Another trace metal study in rats (p. 360); Tissue penetration by ingested mineral fibres? (p. 361); The dimensions of respirable fibreglass (p. 361); Acetaldehyde overexposure (p. 362); Analgesics and the diet (p. 362); Hypersensitivity to chlorocresol (p. 362); Ocular effects of a fluorocarbon (p. 363); Carcinogenicity of nitrosation products of environmental chemicals (p. 363); Fundamental percutaneous absorption studies (p. 364)—NATURAL PRODUCTS: A new fungal food assessed (p. 364); Protective properties of plant fibres (p. 365); Tea-drinkers' anaemia (p. 365)—COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS: A clean slate for zinc pyrithione shampoos (p. 366)—TOXICOLOGY: Contact dermatitis epidemiology (p. 366)—PATHOLOGY: Is zinc polycarboxylate cement tumorigenic? (p. 366); Reactions to sutures in human tissues (p. 367); Leucocyte response to implanted starch (p. 367)—CANCER RESEARCH: A rat model detecting bladder carcinogens (p. 367).

* 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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Research Section

EFFECTS OF CHRONIC ORAL ADMINISTRATION OF ERYTHROSINE IN THE MONGOLIAN GERBIL

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(*Received 28 February 1976*)

Abstract—Approximately equal numbers of male and female Mongolian gerbils, *Meriones unguiculatus*, were given erythrosine (FD & C Red No. 3) by diet and by oral intubation. In the dietary study, four groups evenly divided by sex were treated for 105 wk at levels of 0, 1, 2 or 4%. In the intubation study, four groups were intubated by stomach tube twice weekly for 97 wk with an aqueous solution at levels of 0, 200, 750 or 900 mg/kg. Clinical effects of dietary administration were depression of the haematocrit value, leucocyte count, reticulocyte count and haemoglobin level, and a dose-related loss of weight, ranging from mild in groups fed 1% to marked in groups fed 4%. The dietary study also showed that dose-related changes occurred in the thyroid of animals fed the 1–4% diets. These changes were characterized by the enlargement, with increased storage of colloid, of a majority of the follicles, associated with less prominent but consistent foci of very small follicles (microfollicles) and, in a few animals, focal hyperplasia and intraluminal and interstitial leucocytic infiltration. Although there was a slight increase in follicular size in the thyroids of gerbils given 900 and 750 mg/kg by intubation, a definite effect similar to that in the dietary study was not found. Evaluation of the effect on the liver was complicated by the presence in the controls of multiple granulomas composed primarily of epithelioid cells surrounded by lymphocytes. Although special diagnostic procedures, including stains for micro-organisms, failed to identify the cause, a microbial aetiology was suspected, as this is most common for this type of lesion. In both studies, there was more granulomatosis in the controls than in the treated animals, but in the intubation study there was no evidence of a relation to dose.

INTRODUCTION

Synthetic organic colourings are used in foods, drugs and cosmetics in preference to pigments derived from natural sources because the synthetic dyes usually provide a wider colour range, higher tinctorial power and better light stability. The increasing awareness of possible effects that may be associated with the use of these chemicals has resulted in additional studies of colourings that have hitherto been considered safe. Erythrosine is a colouring widely used in foods, drugs and cosmetics; it was one of the seven colourings originally permitted under the Food and Drug Act of 30 June 1906 (Calvery, 1942) and has been in use since that time. Erythrosine, which is certified by the Food and Drug Administration (FDA) as FD & C Red No. 3, is a bluish-red water-soluble colouring formed by the iodination of fluorescein.

Waliszewski (1952) reported that erythrosine administered orally at 2·5 g/kg body weight or by iv injection at 0·37 g/kg was lethal to mice. After intubation studies in adult male rats, Lu & Lavallée (1964) concluded that the acute oral LD_{50} exceeded 2 g/kg. Hansen, Zwickey, Brouwer & Fitzhugh (1973a) determined the LD_{50} to be 1840 mg/kg in young adult Osborne–Mendel rats given the compound by intubation.

Graham & Allmark (1959) gave sc injections to rats at 250 mg/kg body weight twice daily for 3 days and concluded that the colouring did not affect oestrogenic activity.

In a 2-yr chronic toxicity study in rats fed diets containing from 0·5 to 5·0% erythrosine, Hansen *et al.* (1973a) reported that dietary levels of 5.0% depressed growth, and that spleen-to-body weight ratios were decreased in males at three of the four dose levels and in females at the highest level. In addition, distended caeca were observed in rats fed 1·0% and above, and distension increased with dose level. These investigators also reported that no effects attributable to erythrosine ingestion were seen in beagle dogs after a 2-yr study.

None of the animal experiments thus far reported has shown any evidence that erythrosine may be tumorigenic. No tendency to neoplasia was demonstrated either in the studies reported by Hansen, Davis, Graham, Perry & Jacobson (1973b), in which rats were either fed levels ranging from 0·5 to 4·0% for 86 wk or intubated twice weekly at doses ranging from 100 to 1500 mg/kg for 85 wk, or in 2-yr rat and dog feeding studies (Hansen *et al.* 1973a). Distant tumours were not produced in rats given erythrosine as weekly sc injections for 2 yr, but injection-site ulceration did occur (Hansen *et al.* 1973a; Nelson & Hagan, 1953). After feeding 4·0% erythrosine diets to rats for 12 months, Willheim & Ivy (1953) observed gross staining of the glandular stomach and small in-

testine and granular deposits in the stomach, small intestine and colon, but found no neoplasms. In a short-term toxicity study in rats, Umeda (1956) injected a 5·0% aqueous solution of erythrosine sc; no tumours were produced. In a 500- to 700-day toxicity experiment, Waterman & Lignac (1958) fed the colouring to mice at a level of 1 mg/animal/day and concluded that it had no carcinogenic action.

The pattern of metabolism and excretion of erythrosine in rats was studied by Daniel (1962), who found that 55–72% of the colouring was excreted in the rat faeces within 3 days. Webb, Fonda & Brouwer (1962) showed that the colouring was excreted in an undegraded state primarily by the bile, and that all the colouring could be recovered in the rat excreta within 5 days.

Waliszewski (1952) reported that iv injection of erythrosine into mice produced haemolytic disorders and decreases in numbers of erythrocytes and leucocytes. Bowie, Wallace & Lindstrom (1966) reported reduced haemoglobin and erythrocyte counts in rats after 3 months of oral intubation of erythrosine, but these findings were not confirmed by Hansen *et al.* (1973b).

Elevated concentrations of protein-bound iodine and total iodine in erythrosine-treated rats were reported by Bowie *et al.* (1966) and similar elevations were noted by Hansen *et al.* (1973b), although the latter investigators stated that thyroxine-iodine levels were not affected by treatment.

The present study was performed to test the long-term effects of erythrosine in another species of mammal, the Mongolian gerbil, and to test further the suitability of this animal for laboratory tests as compared with rats and mice. The results of a three-generation reproduction study with gerbils were published previously (Collins, Hansen & Keeler, 1971).

EXPERIMENTAL

Materials and animals. Erythrosine (FD & C Red No. 3), the disodium salt of 9-(*o*-carboxyphenyl)-6-hydroxy-2,4,5,7-tetraiodo-3-isoxanthone (95% pure, USFDA certified as Lot No. X3238), was obtained from H. Kohnstamm & Company, New York. Purina Rat Chow was obtained from the Ralston-Purina Company, St. Louis, Mo. Mongolian gerbils, *Meriones unguiculatus*, were obtained from Tumblebrook Farms, Brant Lake, N.Y. Mature animals, approximately 6 months of age, were used. The animals were housed individually in polyethylene cages with sawdust bedding. Food and water were available *ad lib.*

Treatment. Diets for the feeding experiment were prepared by adding erythrosine to the rat chow at levels of 4·0, 2·0 or 1·0% and blending in a mixer. Three groups, each of 31 gerbils, approximately equally divided by sex, were fed the experimental diets for 105 wk; 64 control gerbils were fed unadulterated rat chow. In the intubation experiment, erythrosine dissolved in water was administered by stomach tube twice weekly for 97 wk to three groups of 40–49 gerbils, approximately equally divided by sex, at dose levels of 200, 750 or 900 mg/kg; a control group of 63 animals was intubated with distilled water only. The dosages were administered in a uniform volume of 10 ml/kg body weight by using an 18-gauge needle with a soldered ball tip.

Tests and observations. The burrowing and hoarding habits of the gerbil prevented the accurate measurement of food consumption. Animal weights and deaths and clinical observations were recorded weekly. At pretreatment and at 3, 6, 12, 18 and 24 months, a minimum of ten animals from each group (at least five of each sex) were lightly anaesthetized with ether, and blood from the midventral vein of the tail of each animal was collected in a spot plate containing EDTA. The only variations in this schedule occurred in the dietary study, where blood was taken at 8 months instead of 6, and in the intubation study, where animals were killed at 22 months instead of 24. Whenever possible, blood was taken from the same animals for successive tests. Haemoglobin level was measured by the cyanmethaemoglobin method with a Fisher haemophotometer. Erythrocytes were counted with a Coulter counter, model F, after dilution with isotonic saline; leucocytes were counted with the same instrument after erythrocytes were lysed with saline containing saponin. A smear count for reticulocytes was done after staining with methylene blue. Packed cell volume was determined by using a microhaematocrit apparatus.

Post-mortem studies. At the termination of the experiments, all gerbils were decapitated and autopsied; heart, liver, spleen, kidneys and testes from all survivors were weighed, and tissues (internal organs of thorax and abdomen, head and one hind leg), fixed in formol-saline, were sent for more detailed gross examination and microscopic study. Of the 157 gerbils started on the feeding experiment, tissues of 146 were stained with haematoxylin and eosin and examined microscopically by the Pathology Division, FDA. The remainder of the animals, which were from all four groups, were discarded chiefly because of advanced autolysis. Microslides included all gross lesions, the heart, lung, liver, kidney and testis from almost all animals, the adrenal, thyroid, brain, eye, spleen, bone (tibio-femoral joint with attached skeletal muscles), trachea, oesophagus, stomach, small intestine, colon, pancreas, uterus and ovary from one half to two thirds of the animals, and the pituitary, parathyroid, skin, urinary bladder, prostate, vesicular gland, cervix uteri, gall bladder, salivary glands and mesenteric lymph node in smaller numbers. In addition to the routine staining with haematoxylin and eosin, the following special histochemical stains were used: Oil Red O for fat, acid-fast for tubercle bacilli and other Mycobacteria, Brown and Brenn's modification of Gram's stain for bacteria, periodic acid–Schiff (with and without digestion), Gomori methenamine silver for fungi, Gridley for fungi, auramine-rhodamine B for fluorescence, mucicarmine for fungi, Giemsa for various micro-organisms, Warthin-Starry for spirochaetes, alizarin red for calcium, Prussian blue for iron, and Ralph for haemoglobin.

Of the 196 gerbils started in the intubation study, 184 were examined microscopically by a private laboratory under contract to the FDA. As in the dietary study, the remaining animals were discarded chiefly because of advanced autolysis. The following tissues were prepared by the haematoxylin and eosin technique for microscopic evaluation: liver, kidneys,

heart, spleen, adrenals, lungs, thyroid, lymph node, testes, prostate, pancreas, ovaries, uterus, parathyroid, pituitary, salivary gland, urinary bladder, trachea, oesophagus, stomach, small intestine, colon, thymus, brain, bone marrow and skeletal muscle. Most of the tissues from the control and high-dose animals but only a smaller proportion from the animals given 200 or 750 mg/kg were examined microscopically. Thyroids from all groups were examined with special care in order to detect possible lesions or changes.

Because pathological changes ranged from minimal to severe, they were assigned numerical values (A. A. Nelson, FDA, personal communication 1955) according to the following scale: none, 0; minimal or minute, 0·25; very small or very slight, 0·5; slight, small or mild, 1·0; slight to moderate, 1·5; moderate, 2·0; moderately severe, 2·5; severe or marked, 3·0; most severe, 4·0. This scale was used in the original pathology report from the dietary study but for the intubation study verbal descriptions of the pathological changes had to be converted to the scale values.

Statistical analysis. Animal mortality was analysed by the chi-square test. Analysis of covariance and a least significant difference (LSD) test were used to compare average weight gains between control and treated animals. Significance for average weight gain was based on means adjusted for initial weight. Analysis of variance and an LSD test were used to compare the control against the treatment groups for each organ-to-body weight ratio. When the variances of each of the group ratios were not homogeneous, a t test was used instead to compare the control with each of the treatment groups. Chi-square and non-parametric Mann–Whitney U tests were used to analyse follicle size and hyperplasia of thyroids and granulomatosis and Kupffer-cell proliferation of livers. Haemoglobin values were analysed by the t test, and erythrocyte, leucocyte and reticulocyte counts and haematocrit values were analysed by the Mann–Whitney U test.

RESULTS

General clinical observations

Diarrhoea with dye in the faeces was observed in treated gerbils. Colouring of the fur could not be detected in the chronic dietary study, possibly because of masking by the natural colour. As expected, pink appeared around the mouths of animals treated by intubation.

Number of deaths

No dose-related increase in deaths of either males or females occurred after ingestion of erythrosine in the diet or by intubation (Table 1). Accidental deaths, discounted as being unrelated to compound dosage, were mostly due to ether overdose during blood sampling or to drowning by inhalation of the compound into the lungs during intubation.

Growth

Although the dye produced no dose-related increase in the death rate, it appeared to be deleterious

Table 1. *Average weight gain and number of deaths of gerbils given erythrosine in the diet or by intubation*

Sex and dose level*	Initial no./group	Average weight gain (g) at month			
		3	6	12	22/24†
		Dietary study			
Males					
0	32	26·0 ± 1·49 (0/32)	23·7 ± 2·00 (0/32)	30·0 ± 2·73 (2/32)	13·1 ± 4·11 (14/31)
1	15	16·1 ± 1·88[g](0/15)	10·7 ± 2·77[g](0/15)	18·1 ± 2·55[c](0/14)	7·5 ± 4·53 (1/14)[a]
2	16	5·2 ± 1·90[i](1/16)	0·0 ± 2·09[i](1/16)	7·5 ± 3·01[i](1/16)	3·9 ± 2·19[i](3/16)
4	15	−4·1 ± 1·79[i](0/15)	−7·3 ± 1·94[i](0/15)	−4·6 ± 3·17[i](1/13)	−9·1 ± 2·68[i](6/13)
Females					
0	32	30.2 ± 1.48 (0/32)	27·2 ± 1·75 (0/32)	32·4 ± 2·88 (0/31)	26·7 ± 3·80 (6/31)
1	16	19·3 ± 1·95[g](0/16)	12·0 ± 2·31[i](0/16)	16·8 ± 2·54[g](0/16)	7·6 ± 4·35[d](4/14)
2	15	4·7 ± 1·10[i](0/15)	2·5 ± 1·50[i](1/15)	6·0 ± 2·10[i](2/15)	0·9 ± 2·08[g](4/15)
4	16	−2·1 ± 1·88[i](1/16)	−3·3 ± 1·77[i](1/16)	−1·1 ± 2·15[i](1/16)	−3·4 ± 3·67[i](4/15)
		Intubation study			
Males					
0	33	22·2 ± 2·41 (1/32)	28·4 ± 3·00 (1/32)	33·1 ± 2·94 (2/31)	26·0 ± 2·84 (8/31)
200	20	15·5 ± 2·14 (0/20)	19·3 ± 3·55 (1/19)	23·8 ± 4·96 (1/18)	12·4 ± 9·02 (9/17)
750	22	19·1 ± 2·53 (1/22)	25·8 ± 3·95 (3/22)	27·6 ± 5·14 (6/21)	18·0 ± 4.51 (12/18)[d]
900	23	18·1 ± 2.45 (0/23)	22·6 ± 3·12 (1/22)	27·6 ± 3·81 (3/21)	20·3 ± 4·30 (7/18)
Females					
0	30	15·2 ± 2·23 (0/30)	21·2 ± 2·47 (3/29)	26·9 ± 2·91 (3/29)	25·4 ± 2·23 (7/27)
200	20	17·2 ± 3·13 (0/20)	20·2 ± 3·48 (1/19)	25·6 ± 4·86 (2/18)	19·2 ± 7·30 (11/17)[d]
750	22	17·6 ± 2·54 (2/22)	22·9 ± 3·32 (2/22)	32·2 ± 2·84 (2/22)	21·2 ± 8·62 (7/17)
900	26	12·2 ± 3·00 (4/26)	16·4 ± 3·16 (4/26)	25·7 ± 4·02 (4/26)	14·8 ± 5·62 (10/20)

*Doses are expressed as percentage in the diet or as mg/kg body weight in the intubation study.
†Values are at 24 months in the dietary study and at 22 months in the intubation study.
Values are expressed as the mean ± SEM of cumulative weight gain. The figures in parentheses are the total no. of animals dead/initial number of animals, both figures being corrected for accidental deaths. Those marked with superscripts differ significantly from the control value: [a]$P < 0·05$; [c]$P < 0·02$; [d]$P < 0·01$; [g]$P < 0·001$; [i]$P < 0·0001$.

to the growth of the gerbils, as shown by the dose-related decrease in body weights of both males and females after the dye-containing diets had been ingested for as short a time as 3 months (Table 1). Body weights were not affected similarly after intubation of the compound (Table 1), possibly because lesser amounts were ingested. The mean body weight of control males at 2 yr of age was very close to that reported by Kramer (1964), but the mean weight of females was nearly 10 g greater.

Organ weights

After dietary ingestion of erythrosine, both males and females had dose-related decreases in the mean weight of the heart; females also had dose-related decreases in liver, spleen and kidney weights (Table 2). In males, weights of liver, spleen, kidney and testis were decreased, but the decreases were not as clearly dose-related. This depression in organ weights appeared to parallel to some extent the depression in overall body weight. Control males in the dietary study had significantly higher organ-to-body weight ratios for heart, liver, and spleen than did males fed the 2 and 4% levels, and control females had significantly higher ratios for liver and spleen than did females fed the 2 and 4% levels. Only the changes in liver-to-body weight ratios in females were dose-related. Table 2 also gives the mean organ weights and organ-to-body weight ratios of males and females in the intubation study. There was no dose-related effect on mean weight of organs in either sex. The spleen presented the greatest variation in weight. The organ-to-body weight ratios obtained in this study showed no dose-related differences in either males or females; they were similar to those obtained by Kramer (1964), except for the liver ratios, which were higher in our study.

Haematology

The haematological values for control gerbils are similar to those obtained by Ruhren (1965) and Mays (1969). Table 3 shows the means and standard errors of these measurements for the diet-fed animals before treatment and at intervals up to 2 yr. The haemoglobin values of males fed the 4% dose level were significantly decreased at 8 and 24 months, and the values for females fed the two highest doses were depressed from 8 months onward. The haematocrit was decreased at 12 and 24 months in females at all three dose levels and in those fed the two highest levels at 8 months but was decreased in males fed the 1% level at 8 months and in those fed the 4% level at 24 months. Only the two highest dose levels caused significant decreases in the number of erythrocytes at 12 months. Erythrocytes were decreased in females fed the 1 and 4% levels at 8 and 24 months, and in those fed all three dose levels at 12 months. Leucocytes were decreased at 24 months in males at all three dose levels and in females at the two highest levels; they were also decreased at 8 months in males fed the 2% dose level and in females fed the 2 and 4% dose levels.

Table 4 shows the haematological values for the gerbils after intubation with erythrosine. Haemoglobin and haematocrit values and leucocyte counts were sporadically affected by the compound. Erythrocyte counts in males at all three dose levels were significantly decreased at 12 and 22 months.

Reticulocyte counts after dietary administration or intubation of erythrosine are given in Table 5. After 24 months, the values for males and females fed the 4% diets were significantly decreased. In the intubation study, no decreases were seen that appeared to be related to treatment. The increase in the males at 750 mg/kg after 12 months was due to a single animal with a reticulocyte count of 25.

Gerbils fed diets containing even moderate amounts of neutral fat or cholesterol are prone to develop high concentrations of serum cholesterol (Clarkson, King & Warnock, 1957; Gordon & Cekleniak, 1960; Gordon, Cekleniak, Stolzenberg, Benitz & Moraski, 1961; Gordon, Stolzenberg & Cekleniak, 1959). In the animals in the present study, plasma of lipaemic appearance was noted equally in both males and females. Lipaemic animals were seen 3, 6 and 12 months after the beginning of the study (actual ages, 9, 12 and 18 months, respectively) but not at pretreatment, 18 months or 24 months (actual ages 6, 24 and 30 months, respectively). This pattern of increase is curious, since the animals were fed the same chow throughout the study without addition of any type of lipid, and indicates a possible metabolic cycle of cholesterol synthesis rather than a consistent increase with age.

General pathological observations

In the dietary study, all animals from the treated groups had viscera and fur-covered skin which were pink to red; the degree of pigmentation varied directly with the dosage level and appeared greater in the gastro-intestinal canal and faeces than in other areas. A similar pattern of pigment deposition was noted in the intubation study, although less colour was present than in the feeding experiment. In both studies, fixation in formol-saline caused pigment to spread diffusely throughout all tissues.

Gross liver pathology in the feeding study

Grossly, the livers of nearly all gerbils were characterized by the presence of a thin, transverse, subcapsular fissure, 1 mm or less in width and depth, extending coronally between the lateral aspects of the ventral portion of the caudal surface of the left lateral and/or median lobe; this fissure was shown microscopically to be a venule. Other changes were most severe in the controls, although they appeared in all four groups. Generalized, irregular, fine-to-coarse nodularity (characterized by slightly projecting nodules 1–10 mm in diameter separated by thin bands 1–2 mm in diameter) was the sole lesion found in the livers of 11 animals (four controls, four fed the 1% diet and three fed the 2% diet). This diffuse nodularity was associated with yellow discoloration in six control animals and two fed the 4% diet, and one of these two also had a few pale, yellowish-white, subcapsular macules of 1–5 mm. Similar scattered macules were also seen in three controls and one animal fed the 1% diet. Focal nodularity, typically involving only the caudal aspect of the median or left lateral lobe near the transverse fissure, appeared in six control animals, 11 animals fed 1%, four fed 2% and three fed 4%. Of the six control animals, livers of four also showed

Table 2. *Organ weights and organ-to-body weight ratios of gerbils given erythrosine in the diet for 24 months or by intubation for 22 months*

Sex and dose level*	No. of gerbils	Heart		Liver		Spleen		Kidneys		Testes	
		Weight (mg)	Ratio (g/kg)	Weight (g)	Ratio (g/kg)	Weight (mg)	Ratio (g/kg)	Weight (g)	Ratio (g/kg)	Weight (g)	Ratio (g/kg)
Dietary study											
Males											
0	17	413·53 ± 13·98	4.19	6.82 ± 0.63	69.16	185·82 ± 29·62	1·88	1·05 ± 0·06	10·69	1·38 ± 0·02	13·98
1	13	381·54 ± 12·81	3·88	6·44 ± 0·47	65·53	118·46 ± 15·97	1·20^{a}	1·01 ± 0·02	10·32	1·40 ± 0·03	14·24
2	13	283·61 ± 11·49	3·37^{d}	3·62 ± 0·26	42·97^{d}	63·15 ± 5·46	0·75^{d}	0·74 ± 0·02	8·77^{c}	1·17 ± 0·03	13·85
4	7	274·28 ± 18·90	3·62^{d}	3·88 ± 0·52	51·31^{a}	67·28 ± 7·81	0·89^{d}	0·74 ± 0·04	9·80	1·11 ± 0·05	14·62
Females											
0	25	392·76 ± 15·20	4·12	7·00 ± 0·59	73·36	178·84 ± 29·32	1·87	0·98 ± 0·06	10·32	—	—
1	10	334·80 ± 19·24	4·09	4·86 ± 0·67	59·40	126·70 ± 42·46	1·55	0·77 ± 0·05	9·44	—	—
2	11	279·27 ± 15·57	3·69^{a}	3·59 ± 0·22	47·46^{d}	58·09 ± 4·96	0·77^{d}	0·70 ± 0·02	9·25	—	—
4	11	273·82 ± 13·85	3·82	3·18 ± 0·36	44·40^{d}	55·45 ± 3·19	0·77^{d}	0·70 ± 0·03	9·74	—	—
Intubation study											
Males											
0	23	409·09 ± 11·23	4·02	7·89 ± 0·66	77·54	192·83 ± 34·94	1·90	1·00 ± 0·04	9·85	1·41 ± 0·02	13·89
200	8	394·12 ± 37·57	4·08	6·34 ± 0·97	65·73	262·75 ± 74·79	2·72	0·97 ± 0·07	10·01	1·26 ± 0·11	13·08
750	6	479·33 ± 15·90	4·59^{a}	7·52 ± 0·33	71·92	576·67 ± 126·02	5·52^{c}	1·59 ± 0·37	15·22	1·37 ± 0·04	13·16
900	11	429·18 ± 15·92	4.13	7·32 ± 0·69	70·43	219·73 ± 49·43	2·11	0·99 ± 0·05	9·57	1·34 ± 0·02	12·94
Females											
0	20	409·90 ± 18·51	4·21	8·63 ± 0·85	88·65	280·15 ± 56·01	2·88	0·98 ± 0·05	10·08	—	—
200	6	412·67 ± 35·88	4·22	8·81 ± 1·16	90·03	407·33 ± 138·92	4·16	0·98 ± 0·09	9·98	—	—
750	10	416·90 ± 17·66	4·19	7·02 ± 0·74	70·67	139·90 ± 16·83	1·41^{a}	1·01 ± 0·04	10·21	—	—
900	10	404·60 ± 22·70	4·14	6·87 ± 0·79	70·32	218·50 ± 39·27	2·24	0·97 ± 0·06	9·94	—	—

*Doses are expressed as percentage in the diet or as mg/kg body weight in the intubation study.

Weight values are means ± SEM. Ratios marked with superscripts are significantly different from the control ratio: $^{a}P < 0·05$; $^{c}P < 0·02$; $^{d}P < 0·01$.

yellow discoloration. Single changes included yellow discoloration in eight controls, focal red and tan mottling in one animal at each dose level of 0, 1 and 2%, and a few foci, which were 1–4 mm in diameter, slightly depressed, subcapsular, linear or stellate and red to reddish-brown, in three controls and in one and two animals fed the 1 and 2% diets, respectively.

Microscopic liver pathology in the feeding study

Microscopically the livers of these animals showed that overall liver damage in both males and females was in reverse proportion to the dose (Table 6). The major abnormalities in these livers, in order of importance, were granulomatosis, Kupffer-cell proliferation, fatty metamorphosis and nodularity.

As Table 6 indicates, granulomatosis showed a more consistent negative relation to dose in males than in females. Grades in individual animals ranged from minimal to moderately severe, with the incidence of higher grades likewise varying inversely with dose level. The granulomatosis was characterized by the presence of multiple lesions that were generally 0·1 to 0·3 mm in diameter but ranged up to 1 mm (Fig. 1). Most were round and discrete, and consisted of concentrically arranged epithelioid cells often surrounded by a periphery of lymphocytes (Fig. 2). Some, however, tended to coalesce or to form streaks and to show a more irregular cell pattern with additional cell types, chiefly plasma cells, but occasionally neutrophils also. Necrosis was present in granulomas in

Table 3. *Haemoglobin, haematocrit and erythrocyte and leucocyte counts in gerbils fed erythrosine in the diet*

Sex and dose level (%)	Haematological data at month			
	0	8	12	24
		Haemoglobin (g/100 ml)		
Males				
0	11·8 ± 0·50 (10)	12·8 ± 0·22 (10)	12·9 ± 0·59 (10)	12·7 ± 0·54 (10)
1	13·5 ± 0·47^{a}(5)	12·2 ± 0·21^{a}(10)	14·1 ± 0·22 (10)	13·5 ± 0·33 (10)
2	12·5 ± 0·37 (5)	12·4 ± 0·27 (10)	13·2 ± 0·18 (10)	12·9 ± 0·22 (10)
4	13·4 ± 0·35^{c}(5)	12·0 ± 0·19^{c}(10)	13·1 ± 0·36 (10)	11·3 ± 0·28^{a}(7)
Females				
0	13·3 ± 0·50 (9)	12·6 ± 0·17 (10)	13·9 ± 0·46 (10)	12·8 ± 0·37 (10)
1	12·4 ± 1·17 (5)	11·7 ± 0·46 (10)	12·8 ± 0·29 (10)	11·6 ± 0·65 (10)
2	11·8 ± 0·43 (5)	10·7 ± 0·30^{g}(10)	11·9 ± 0·28^{e}(10)	11·1 ± 0·34^{e}(10)
4	12·3 ± 0·96 (5)	10·8 ± 0·42^{d}(10)	11·6 ± 0·50^{e}(10)	11·1 ± 0·31^{e}(10)
		Haematocrit (%)		
Males				
0	36·1 ± 1·16 (8)	36·6 ± 0·58 (10)	40·2 ± 1·49 (10)	39·0 ± 1·08 (10)
1	41·2 ± 1·93^{b}(5)	34·3 ± 0·62^{c}(10)	42·1 ± 0·79 (10)	41·5 ± 0·79 (10)
2	39·7 ± 1.86 (3)	37·4 ± 1·17 (10)	40·4 ± 0·62 (10)	41·0 ± 0·60 (10)
4	42·2 ± 1·93^{b}(4)	35·8 ± 0·80 (10)	38·3 ± 1·13 (10)	34·4 ± 1·34^{a}(7)
Females				
0	44·2 ± 1·94 (10)	35·8 ± 0·65 (10)	44·9 ± 1·77 (10)	41·2 ± 0·76 (10)
1	38·3 ± 3·84 (3)	33·4 ± 1·47 (10)	37·6 ± 1·19^{a}(10)	36·0 ± 2·01^{c}(10)
2	44·5 ± 1·50 (4)	31·9 ± 1·25^{c}(9)	37·7 ± 1·19^{f}(9)	35·3 ± 0·86^{f}(10)
4	46·7 ± 0·88 (3)	31·6 ± 0·97^{c}(10)	36·4 ± 1·36^{f}(10)	35·2 ± 0·89^{f}(10)
		Erythrocytes (10^6/mm^3)		
Males				
0	6·9 ± 0·32 (10)	7·2 ± 0·10 (10)	8·2 ± 0·53 (10)	6·1 ± 0·27 (10)
1	7·8 ± 0·19^{a}(5)	6·9 ± 0·15 (10)	7·0 ± 0·21 (10)	6·1 ± 0·12 (10)
2	7·3 ± 0·15 (5)	7·1 ± 0·15 (10)	6·6 ± 0·11^{a}(10)	5·4 ± 0·19 (10)
4	8·0 ± 0·24^{c}(5)	7·0 ± 0·09 (10)	5·9 ± 0·16^{e}(10)	5·6 ± 0·10 (7)
Females				
0	8.6 ± 0·34 (10)	7·3 ± 0·11 (10)	8·2 ± 0·42 (10)	6·1 ± 0·16 (10)
1	7·1 ± 0·84^{a}(5)	6·6 ± 0·22^{a}(10)	6·2 ± 0·14^{g}(10)	4·9 ± 0·26^{d}(10)
2	7·3 ± 0·19^{c}(5)	6·8 ± 0·23 (10)	6·2 ± 0·10^{g}(10)	5·6 ± 0·25 (10)
4	7·7 ± 0·53 (5)	6·6 ± 0·20^{d}(10)	5·5 ± 0·27^{g}(10)	5·1 ± 0·14^{g}(10)
		Leucocytes		
Males				
0	12,470 ± 1028 (10)	13,130 ± 1810 (10)	14,375 ± 1889 (8)	11.210 ± 896 (10)
1	10,600 ± 1038 (5)	10,210 ± 936 (10)	15,240 ± 1668 (10)	7,360 ± 681^{d}(10)
2	14,260 ± 1823 (5)	8,360 ± 675^{a}(10)	11,190 ± 1091 (10)	6.150 ± 744^{e}(10)
4	12,460 ± 1623 (5)	10,910 ± 1129 (10)	16,425 ± 1591 (8)	6,143 ± 713^{e}(7)
Females				
0	14,443 ± 1142 (7)	12,110 ± 1063 (10)	19,490 ± 4764 (10)	8,900 ± 803 (10)
1	6,620 ± 1122^{d}(5)	10,050 ± 604 (8)	15,500 ± 1788 (10)	7.760 ± 893 (10)
2	9,100 ± 1667^{a}(5)	7,880 ± 970^{c}(10)	13,350 ± 1807 (10)	5.790 ± 645^{e}(10)
4	6,380 ± 850^{g}(5)	7.767 ± 1122^{d}(9)	13.940 ± 2176 (10)	5.440 ± 316^{g}(10)

Values are expressed as the mean ± SEM for the numbers of gerbils indicated in parentheses. Those marked with superscripts differ significantly from the control values: $^{a}P < 0·05$; $^{b}P < 0·025$; $^{c}P < 0·02$; $^{d}P < 0·01$; $^{e}P < 0·005$; $^{f}P < 0·002$; $^{g}P < 0·001$.

Table 4. *Haemoglobin, haematocrit and erythrocyte and leucocyte counts in gerbils given erythrosine by intubation twice weekly*

Sex and dose level (mg/kg)	Haematological data at month			
	0	6	12	22
		Haemoglobin (g/100 ml)		
Males				
0	11·7 ± 0·46 (10)	13·2 ± 0·22 (10)	14·3 ± 0·10 (10)	13·3 ± 0·20 (10)
200	12·1 ± 0·37 (5)	13·0 ± 0·23 (10)	14·0 ± 0·17 (10)	12·9 ± 0·42 (8)
750	12·4 ± 0·51 (5)	13·9 ± 0·42 (10)	13·8 ± 0·60 (10)	12·1 ± 0·46[c] (6)
900	11·9 ± 0·28 (5)	13·4 ± 0·39 (9)	13·9 ± 0·49 (10)	12·7 ± 0·51 (10)
Females				
0	12·1 ± 0·36 (10)	13·1 ± 0·42 (10)	13·8 ± 0·23 (10)	13·3 ± 0·26 (10)
200	12·9 ± 0·13 (5)	12·3 ± 0·30 (10)	13·7 ± 0·26 (10)	12·1 ± 0·89 (6)
750	12·5 ± 0·34 (5)	12·4 ± 0·18 (10)	13·5 ± 0·12 (10)	13·8 ± 0·38 (10)
900	12·1 ± 0·40 (5)	12·8 ± 0·29 (10)	13·9 ± 0·26 (10)	13·1 ± 0·52 (10)
		Haematocrit (%)		
Males				
0	36·0 ± 1·51 (9)	38·0 ± 0·47 (10)	43·6 ± 0·78 (10)	41·4 ± 0·69 (10)
200	38·2 ± 1·31 (4)	36·7 ± 0·52 (10)	42·9 ± 0·48 (10)	41·0 ± 1·27 (8)
750	36·5 ± 2·33 (4)	37·8 ± 1·52 (9)	40·9 ± 0·97 (10)	39·7 ± 1·28 (6)
900	37·7 ± 2·50 (4)	36·1 ± 0·50[a] (10)	41·3 ± 1·27 (10)	39·2 ± 1·00 (10)
Females				
0	36·4 ± 1·44 (10)	36·4 ± 1·02 (10)	41·2 ± 0·68 (10)	40·3 ± 0·70 (10)
200	40·2 ± 1·07 (5)	35·6 ± 1·07 (10)	42·1 ± 0·90 (10)	41·0 ± 2·44 (6)
750	37·6 ± 1·33 (5)	35·3 ± 0·54 (10)	41·8 ± 0·49 (10)	42·4 ± 1·07 (10)
900	36·0 ± 2·43 (5)	35·4 ± 1·26 (10)	40·7 ± 0·80 (10)	40·0 ± 1·49 (10)
		Erythrocytes (10^6/mm^3)		
Males				
0	7·0 ± 0·25 (10)	7·2 ± 0·11 (10)	7·4 ± 0·13 (10)	7·1 ± 0·18 (10)
200	7·2 ± 0·46 (5)	7·2 ± 0·10 (10)	6·7 ± 0·07[g] (10)	6·1 ± 0·32[c] (8)
750	7·5 ± 0·24 (5)	7·6 ± 0·20 (10)	5·7 ± 0·17[e] (10)	6·0 ± 0·21[c] (6)
900	7·3 ± 0·25 (5)	7·1 ± 0·15 (10)	6·1 ± 0·17[g] (10)	6·1 ± 0·22[d] (10)
Females				
0	7·7 ± 0·26 (10)	7·6 ± 0·15 (10)	7·2 ± 0·13 (10)	6·6 ± 0·34 (10)
200	7·8 ± 0·30 (5)	7·4 ± 0·16 (10)	7·2 ± 0·22 (10)	5·9 ± 0·63 (6)
750	7·6 ± 0·26 (5)	7·3 ± 0·12 (10)	7·0 ± 0·23 (10)	6·7 ± 0·27 (10)
900	7·7 ± 0·33 (5)	7·1 ± 0·17[a] (10)	7·1 ± 0·16 (10)	6·3 ± 0·32 (10)
		Leucocytes		
Males				
0	13,340 ± 893 (10)	10,850 ± 942 (10)	14,050 ± 1072 (10)	11,570 ± 684 (10)
200	12,960 ± 1378 (5)	12,811 ± 893 (9)	19,250 ± 2133[a] (10)	9,962 ± 1162 (8)
750	12,260 ± 1011 (5)	11,200 ± 847 (10)	15,180 ± 1283 (10)	11,383 ± 1269 (6)
900	12,040 ± 717 (5)	15,060 ± 1621 (10)	15,250 ± 1662 (10)	13,260 ± 1301 (10)
Females				
0	13,390 ± 938 (10)	13,187 ± 1241 (8)	15,330 ± 1378 (10)	12,050 ± 1301 (10)
200	12,200 ± 1725 (5)	12,310 ± 1224 (10)	15,640 ± 2136 (10)	9,983 ± 1888 (6)
750	12,300 ± 1322 (5)	12,022 ± 1088 (9)	15,150 ± 1139 (10)	12,980 ± 1402 (10)
900	11,640 ± 536 (5)	13,987 ± 2101 (8)	13,920 ± 916 (10)	11,460 ± 572 (10)

Values are expressed as the mean ± SEM for the numbers of gerbils indicated in parentheses. Those marked with superscripts differ significantly from the control value: [a]$P < 0.05$; [c]$P < 0.02$; [d]$P < 0.01$; [e]$P < 0.005$; [g]$P < 0.001$.

only six gerbils (four controls and two fed the 2% diet), with typical caseation necrosis in three of these six. Small amounts of pale yellow acid-fast ceroid pigment, also present in very slight degree in the cytoplasm of macrophages occurring in small foci scattered through almost all the livers, were seen in many granulomas, as were occasional deposits of calcium (diagnosed by an alizarin red stain) and a crystalline material. In attempting to determine the aetiology of the granulomas, cultures could not be obtained, as no unfixed tissue remained. However, special stains for fungi, acid-fast bacilli, other bacteria, spirochaetes and other micro-organisms were performed on a considerable number of affected livers. No micro-organisms could be identified by any of the above techniques.

Kupffer-cell proliferation was seen either in streaks or in patches, largely periportal; only the proliferation of recognizable Kupffer cells, as opposed to that in granulomas in which the epithelioid cells undoubtedly were derived from Kupffer cells, was graded. The intensity in individual livers ranged from minimal to moderate (0·25–2 on the scale), and the proliferation was more prominent in treated animals than in controls.

Changes that were less prominent but that, together with granulomatosis, led to architectural irregularity and nodularity are considered together. The most im-

Table 5. *Reticulocyte counts (% of erythrocytes) in gerbils given erythrosine in the diet or by intubation*

Sex and dose level*	Values at month 0	6/8†	12	22/24‡
Dietary study				
Males				
0	1·1 ± 0·27 (9)	1·7 ± 0·26 (10)	2·7 ± 0·70 (10)	1·6 ± 0·95 (10)
1	1·6 ± 0·60 (5)	0·7 ± 0·16[c] (10)	1·6 ± 0·19 (10)	0·6 ± 0·27 (10)
2	0·8 ± 0·34 (5)	1·4 ± 0·15 (10)	1·5 ± 0·24 (10)	0·3 ± 0·12 (10)
4	1·0 ± 0·41 (5)	1·4 ± 0·21 (10)	1·7 ± 0·28 (10)	0·1 ± 0·10[a] (7)
Females				
0	0·9 ± 0·15 (10)	2·1 ± 0·26 (10)	2·0 ± 0·32 (10)	0·7 ± 0·13 (10)
1	1·1 ± 0·22 (4)	1·4 ± 0·15[a] (10)	1·5 ± 0·18 (10)	0·8 ± 0·54 (10)
2	1·3 ± 0·51 (4)	1·3 ± 0·21[c] (10)	1·1 ± 0·16 (10)	0·6 ± 0·33 (10)
4	1·4 ± 0·50 (3)	1·0 ± 0·17[f] (10)	2·0 ± 0·65 (10)	0·2 ± 0·15[c] (10)
Intubation study				
Males				
0	1·8 ± 0·27 (10)	1·9 ± 0·20 (10)	2·0 ± 0·26 (10)	0·8 ± 0·30 (10)
200	1·9 ± 0·44 (5)	1·4 ± 0·13 (10)	1·9 ± 0·15 (10)	1·6 ± 0·64 (8)
750	2·1 ± 0·29 (5)	1·2 ± 0·14[c] (10)	7·6 ± 1·21 (10)	0·5 ± 0·24 (6)
900	1·2 ± 0·35 (5)	1·8 ± 0·39 (10)	1·9 ± 0·12 (10)	1·9 ± 0·96 (10)
Females				
0	2·3 ± 0·39 (10)	1·1 ± 0·18 (10)	1·7 ± 0·14 (10)	1·2 ± 0·49 (10)
200	1·1 ± 0·26[a] (5)	1·1 ± 0·16 (10)	2·1 ± 0·18 (10)	0·6 ± 0·19 (6)
750	1·7 ± 0·41 (5)	1·5 ± 0·21 (10)	1·9 ± 0·19 (10)	0·6 ± 0·40 (10)
900	0·8 ± 0·22[c] (5)	1·7 ± 0·15[a] (10)	1·9 ± 0·22 (10)	1·1 ± 0·81 (10)

*Doses are expressed as percentage in the diet or as mg/kg body weight in the intubation study.
†Values at 8 months in the dietary study and 6 months in the intubation study.
‡Values at 24 months in the dietary study and 22 months in the intubation study.
Values are expressed as the mean ± SEM for the numbers of gerbils indicated in parentheses. Those marked with superscripts differ significantly from the control: [a]$P < 0·05$; [c]$P < 0·02$; [f]$P < 0·002$.

portant was fatty metamorphosis, which was, in general, inversely proportional to the dose; the average range was from slight to moderate in the controls to very slight in animals fed the 2 and 4% diets. Other changes observed were minimal to small amounts of focal hepatic cell enlargement and necrosis, variation in hepatic cell size, non-granulomatous leucocytic infiltration (generally periportal or associated with fibrosis and focal necrosis) and fibrosis (in streaks or irregularly disposed subcapsular foci). These changes were seen in all groups but tended to be more severe in the controls and in animals fed the lower dose levels.

Nodularity ranged from mild to severe and was caused by thin bands of fibrous tissue, Kupffer cells, leucocytes and small veins and capillaries coursing

Table 6. *Liver disease in gerbils given erythrosine in the diet or by intubation*

Mode of administration and dose level	No. of livers microsectioned M	No. of livers microsectioned F	Grade of overall pathology* M	Grade of overall pathology* F	Granulomatosis: Animals affected (%) M	Animals affected (%) F	Average grade* M	Average grade* F	Animals with grade 1 or more (%)* M	Animals with grade 1 or more (%)* F	Animals with grade 2 or more (%)* M	Animals with grade 2 or more (%)* F	Grade of Kupffer cell proliferation* M	Grade of Kupffer cell proliferation* F
Diet (%)														
0	30	29	1·4†	1·3	80·0	79·3	0·8	0·6	30·0	27·6	16·7	3·4	0·1†	0·4
1	15	15	1·2	1·0	66·7	53·3	0·5	0·4	13·3	13·3	13·3	13·3	0·6[g]	0·5
2	14	13	0·6[d]	0·6[d]	85·7	46·2	0·3	0·2[a]	7·1	7·7	0	0	0·2	0·5
4	13	15	0·7[d]	0·7[c]	46·2	53·3	0·1[c]	0·3	0	13·3	0	6·7	0·5	0·5
Intubation (mg/kg)														
0	26	27	Not measured		42·3	29·6	0·9	0·7	42·3	29·6	34·6	18·5	Not measured	
200	12	11			16·7	27·3	0·3	0·4	16·7	18·2	16·7	9·1		
750	12	11			41·7	36·4	0·5	0·2	33·3	9·1	16·7	0		
900	22	25			27·3	24·0	0·2	0·4	9·1	20·0	4·5	16·0		

*See Experimental section for listing of grades.
†Grade is based on 29 animals only.
Values marked with superscripts differ significantly from the control: [a]$P < 0·05$; [c]$P < 0·02$; [d]$P < 0·01$; [e]$P < 0·005$; [g]$P < 0·001$.

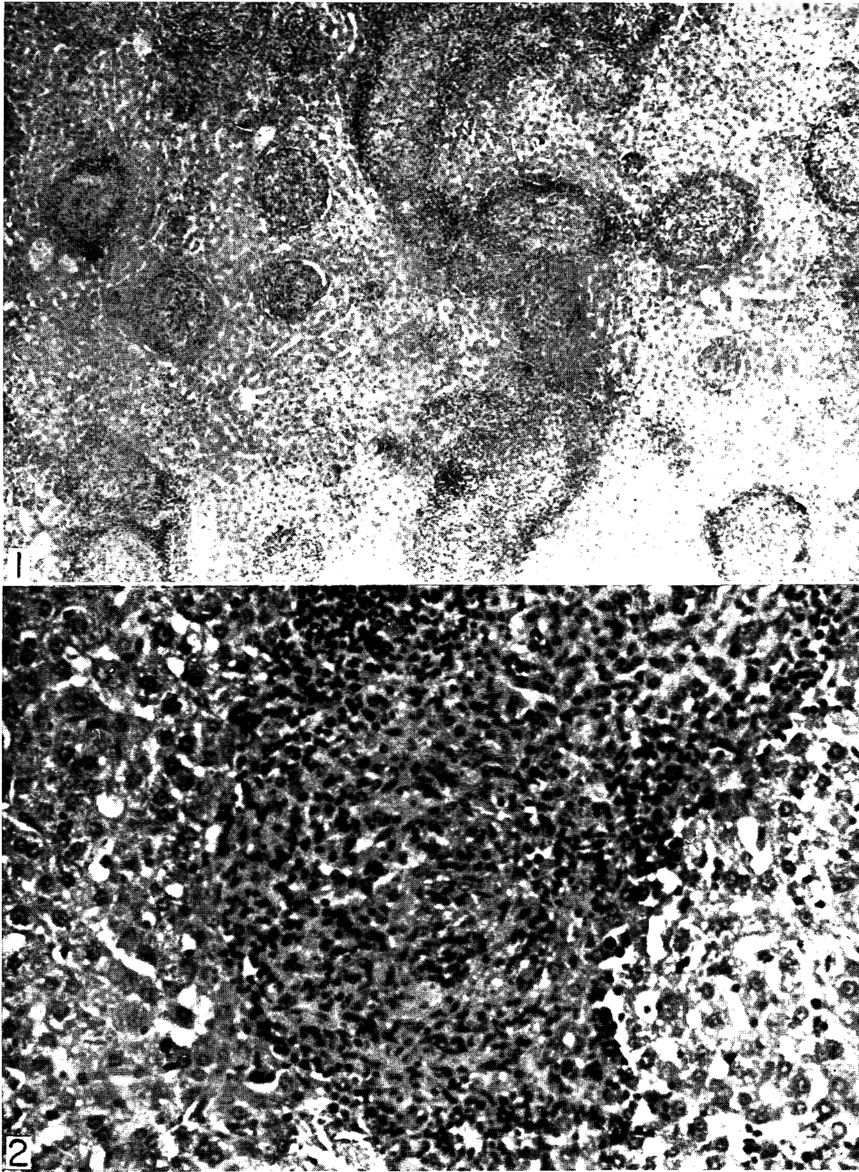


Fig. 1. Liver of an untreated (control) gerbil showing multiple granulomas. Most are discrete, but some are confluent. Haematoxylin and eosin × 47.

Fig. 2. Higher magnification of hepatic granuloma in Fig. 1, characterized by a centre of epithelioid cells surrounded by a periphery composed largely of lymphocytes. Haematoxylin and eosin × 188.

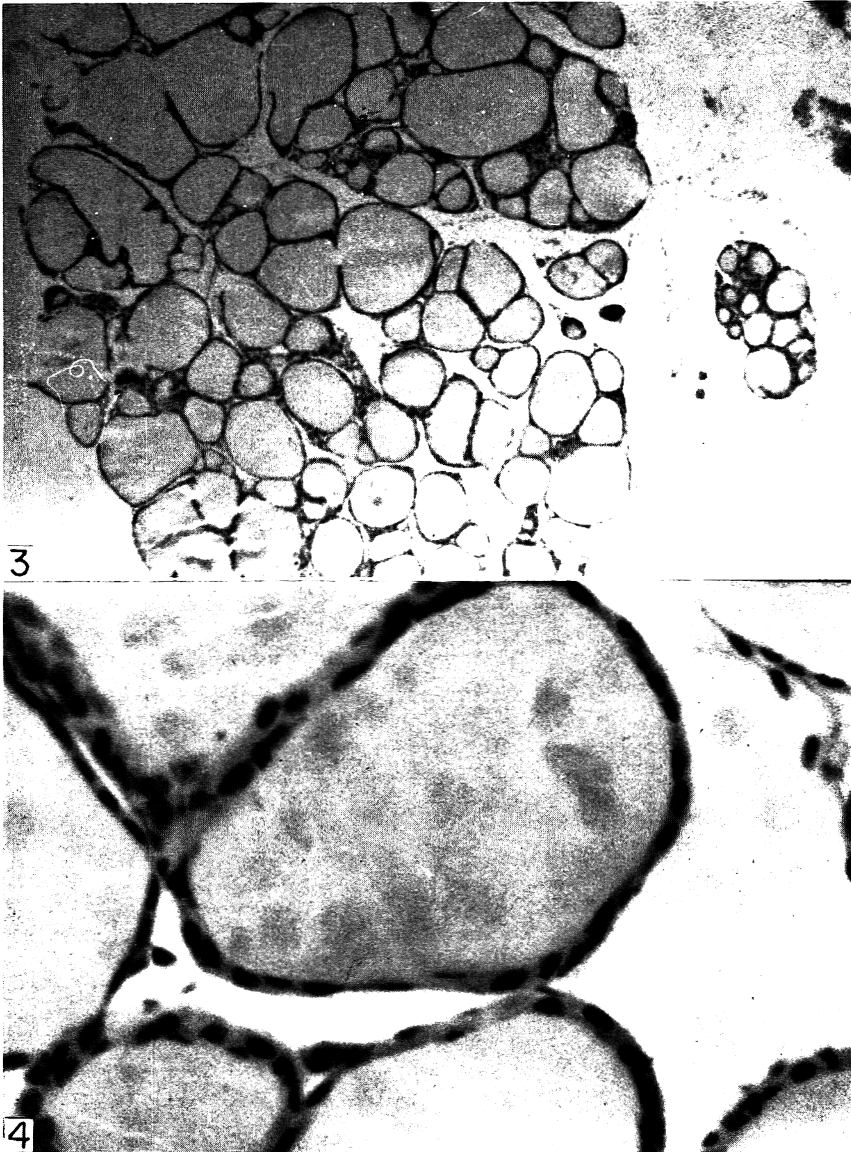


Fig. 3. Thyroid of untreated gerbil, showing follicles varying little in size. Haematoxylin and eosin × 47.

Fig. 4. High magnification of thyroid in Fig. 3, showing follicles lined by simple squamous epithelium and lumina filled with abundant, largely homogeneous colloid. Haematoxylin and eosin × 469.

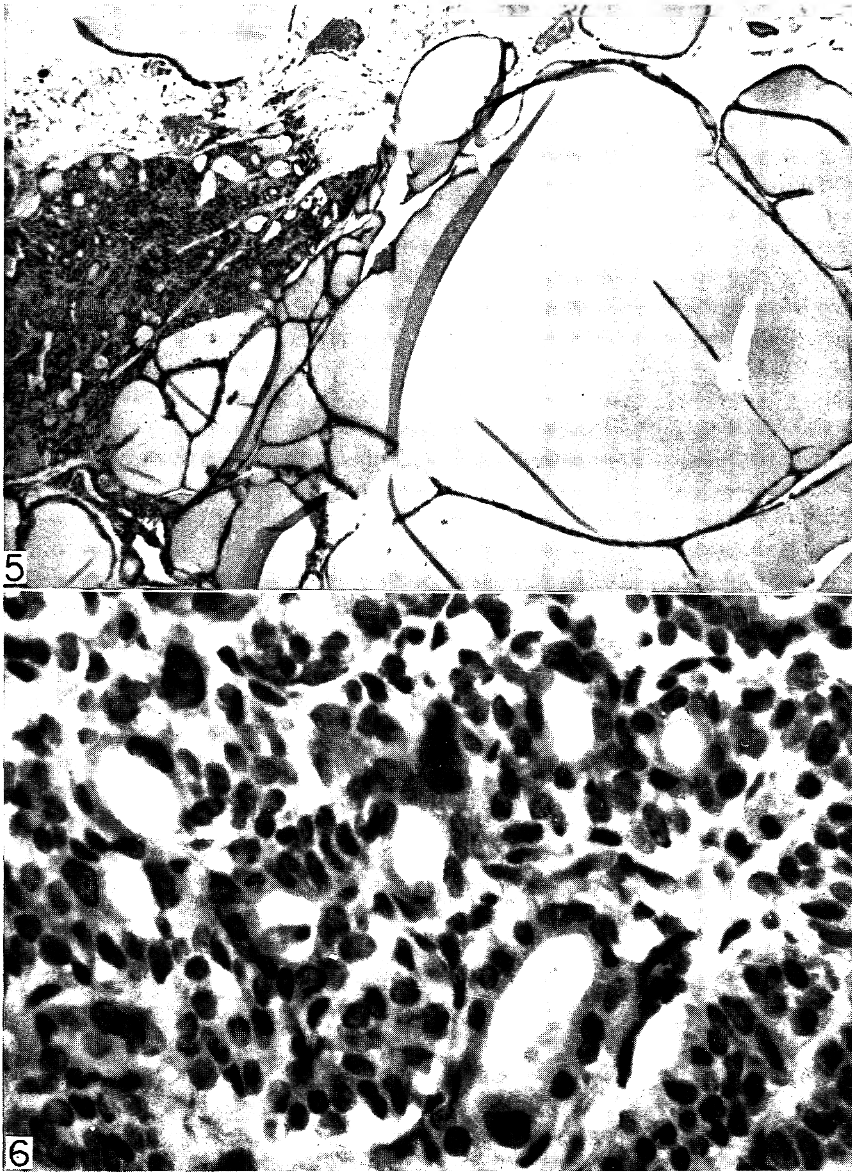
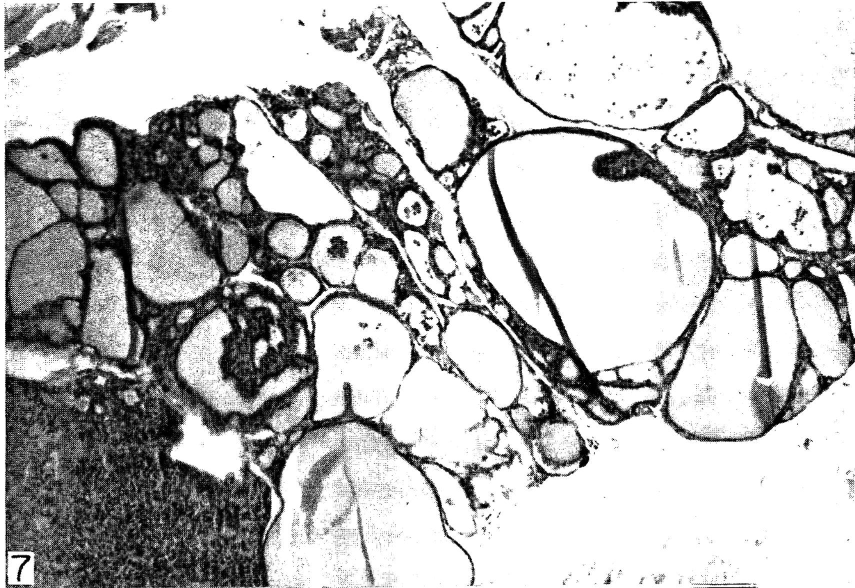


Fig. 5. Thyroid of gerbil fed 4% erythrosine, with follicles varying in size from those distended with colloid and over five times as large as the largest in the control tissue (Fig. 3) to very small ones with little or no colloid (in the dark-staining area). Haematoxylin and eosin × 47.

Fig. 6. Dark-staining area from thyroid in Fig. 5, consisting largely of small follicles lined by simple squamous to simple cuboidal epithelium with very small to pinpoint lumina either empty or containing sparse, lumpy colloid; a few interstitial parafollicular cells are also evident. Haematoxylin and eosin × 469.



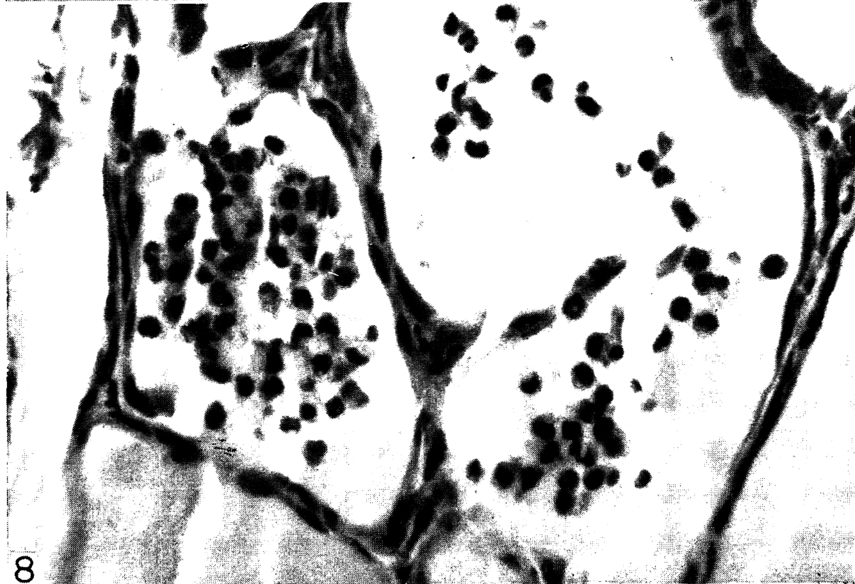


Fig. 7. Thyroid of gerbil fed 1% erythrosine, showing some variation in size and enlargement of follicles and, in addition, foci of hyperplasia characterized by intraluminal invagination of papillae covered by proliferating columnar epithelium, focal intraluminal infiltration of leucocytes and at one side, the parathyroid. Haematoxylin and eosin × 47.

Fig. 8. Thyroid of another gerbil fed 1% erythrosine showing intraluminal leucocytic infiltration. Haematoxylin and eosin × 469.

between central veins, between portal veins, from portal to central veins or irregularly to encircle groups of hepatic cells. It was found in a total of 16 gerbils (seven controls, four fed 1%, four fed 2% and one fed 4%). However, it was accompanied by good evidence of focal nodular regeneration of hepatic cells, a feature required in addition to this type of fibrosis for a diagnosis of true cirrhosis, in only three animals (one control male, one male fed 1% and one female fed 2%). Only one tumour, a benign hepatic cell adenoma in a control male, was found, and the livers of two females, one fed 1% and one fed 4%, were the sites of single foci of adenomatoid hyperplasia.

Liver pathology in the intubation study

The livers of gerbils in the intubation experiment showed no deleterious effect attributable to erythrosine. Granulomatosis, the major hepatic lesion in the dietary study, was also the chief lesion seen in the livers from the intubation study; its incidence and grade in the latter study are given in Table 6. As in the dietary study, the average grade of granulomatosis with intubation was greater in the control than in the treated males, the trend being less definite in the females. However, unlike the situation in the feeding experiment, the reduction in the erythrosine-intubated animals showed no relation to dose, and although there was a difference in degree between treated and control groups, there was no significant difference in incidence. Hepatic lesions that displayed no relation to the presence or absence of erythrosine were hepatic cell vacuolation, which averaged slight to moderate in both control and treated groups, and minimal to slight amounts of focal necrosis, bile-duct proliferation, focal hepatic cell hyperplasia and cystic change, which were seen occasionally in animals in all four groups.

Thyroid pathology in the feeding study

The only gross abnormality noted was enlargement of each lateral lobe to a diameter of 3 and 4 mm in one male and one female, respectively, fed the 4% dietary level. In the controls (Figs 3 & 4) the follicles were uniform in size, averaging 0·2 mm in diameter with a range of 0·1–0·9 mm, lined by simple squamous to low simple cuboidal epithelium and filled with homogeneous, eosinophilic colloid; only rarely were smaller follicles found. As these thyroids resemble those of other rodents, they may be considered within normal limits. A pathological change reminiscent of nodular goitre in man and showing a definite, though imperfect, linear relation to dose was seen in both sexes at all three levels of treatment. Most of the thyroid follicles in the treated gerbils were greatly enlarged, lined by simple squamous epithelium and distended with homogeneous, eosinophilic colloid (Fig. 5), although foci of follicles smaller than those in the controls and comprising 5–60% (20% average) of the total were also consistently present (Fig. 5). Table 7 shows the microscopic changes in the thyroid glands of the gerbils fed erythrosine. Both the average and maximum diameters of the large follicles increased with increasing dose levels, the maximum measurements being 0·9, 1·4, 1·8 and 2·5 mm with dietary levels of 0, 1, 2 and 4%, respectively, with corresponding average diameters of 0·2, 0·4, 0·5 and 0·6 mm in the male groups and 0·2, 0·2, 0·6 and 0·4 mm in the females. The number of animals showing follicular enlargement of grade 1 (slight) or more was also significantly increased at all three dose levels in the dietary study (Table 7). The small follicular change (Fig. 6), which ranged in grade from 1 to 2·5, was seen in two, six and 11 animals at dose levels of 1, 2 and 4%, respectively, and was characterized by follicles measuring 0·1 mm or less in diameter, lined by epithelium that was usually simple cuboidal (but squamous or columnar in a few) and containing lumina that were either empty or filled with thin, vacuolated, pale colloid. Thyroids of some treated animals also contained foci with medium to large follicles lined by proliferating epithelial cells, which increased in height from the normal squamous or low

Table 7. *Comparison of different methods of oral administration of erythrosine in inducing effects on the thyroid*

Mode of administration and dose level	Thyroids microsectioned		Enlargement of follicles									Hyperplasia			
			Average grade*		Maximum grade*	Number showing grade*						M		F	
						1 or more		2 or more		3 or more					
	M	F	M	F		M	F	M	F	M	F	No.	Grade range*	No.	Grade range*
Diet (%)															
0	17	18	0·0	0·0	0	0	0	0	0	0	0	0	0·0	0	0·0
1	8	13	0·5	0·5^{a}	2	3^{a}	6^{c}	1	0	0	0	1	1·0	1	1·5
2	10	12	0·8^{c}	1·1^{f}	2	7^{h}	10^{i}	0	3	0	0	3	1·0	3	1·0
4	9	11	1·2^{f}	1·0^{c}	3	8^{i}	7^{h}	3	2	0	2	5	1·0–1·5	6	1·0–2·0
Intubation (mg/kg)															
0	20	19	0·9	0·5	2	14	7	4	3	0	0	0	0·0	0	0·0
200	13	14	0·8	1·0	2	6	10	4	4	0	0	1	1	1†	0·25
750	17	18	1·8^{f}	1·4^{c}	2	16	13	14^{g}	13^{f}	0	0	1	2·0	3	0·25–1†
900	18	22	1·3	1·3^{c}	2	14	16^{a}	10	12^{c}	0	0	0	0·0	2	1†

*See Experimental section for listing of grades.
†Focal.
Values marked with superscripts differ significantly from the control: $^{a}P < 0.05$; $^{c}P < 0.02$; $^{e}P < 0.005$; $^{f}P < 0.002$; $^{g}P < 0.001$; $^{h}P < 0.0005$; $^{i}P < 0.0001$.

cuboidal to columnar and showed focal papillary invagination into the lumina (Fig. 7); this change and the small follicles with high cuboidal to columnar epithelium are characteristic of hyperplasia (Hazard, 1971; Sommers, 1971). Such hyperplasia (Table 7) was seen in the same animals with the small follicle change. These changes may be attributed to erythrosine. Another alteration with evidence of an increase in the treated groups was slight focal infiltration of follicular lumina with occasional interstitial infiltration of macrophages, lymphocytes, and neutrophils (Fig. 8), which was found in three (female) controls and in five males and three females, three males and four females, and five females fed the 1, 2 and 4% diets, respectively.

Thyroid pathology in the intubation study

Table 7 compares thyroid pathology in the intubation study with that observed in the feeding study. Measurements of diameter of the lumina of the follicles were not recorded for the intubation study as they were for the dietary study, but grading of the degree of follicular enlargement in both made comparisons between the two possible. Although the positive relation between increasing dose and increase in follicular size observed in the dietary study was not seen in the intubation study, there was an increase in the number of animals given 750 and 900 mg/kg showing follicular enlargement of grade 2 or more. However, no increase was observed in maximum grade, and the variability of control values for average grade and the similarity of the average grades at the highest dose levels of both studies suggested a low order of correlation. As in the dietary experiment, thyroid hyperplasia was not seen in any of the controls, and its low incidence, low grade (only slight except for one male in which it was moderate in degree) and lack of any real relation to dose in the groups intubated with erythrosine decreased the likelihood that it was attributable to administration of the colouring. The small follicle change characteristic of the thyroids in the gerbils treated by diet was not evident in those of the animals treated by intubation.

Lymph node pathology

Grossly, mesenteric lymph nodes in the majority of gerbils in the dietary study were oval, reddish-brown and not over 0·3 cm in diameter, i.e. barely visible to the naked eye. In 13 males and two female control animals, one animal of each sex fed 1%, two males and one female fed 2% and one of each sex fed 4%, one or more nodes were enlarged to 2·5 × 1 × 1 cm and showed red to black discoloration. The largest nodes were found in the control group. Microscopic examination showed that nearly all the grossly enlarged and discoloured mesenteric nodes had changes characteristic of a process in the mouse known as mesenteric disease (Dunn, 1954; Simonds, 1925). In these gerbils the most prominent features were sinusoidal congestion and haemorrhage, erythrocytophagocytosis and macrophagic haemosiderosis, a change which was undoubtedly secondary to erythrocytophagocytosis. Other changes, which were less noticeable in this group of gerbils than in mice, were haematopoiesis and atrophy of lymph follicles; significant amounts (grade 1 or more) were found in 11 controls (ten males) and in four, two and three animals fed the 1, 2 and 4% diets, respectively. Moreover, mesenteric disease was three times as severe in the male controls as in any other group, averaging slight to moderate in control males, and very slight in control females and in both sexes fed the 2 and 4% diets; none was apparent in animals fed the 1% diet.

There was no evidence of an effect of treatment on other lymph nodes in the animals in the feeding study. Changes similar to those in the mesenteric nodes of gerbils in the feeding study were noticed in the lymph nodes of a few gerbils in the intubation experiment, but no relation to the administration of erythrosine was detectable.

Spleen pathology in the dietary study

Normal weight and measurements of the spleen were difficult to ascertain because of our lack of experience with the gerbil, the depression in body weight in all three treated groups which resulted in a concomitant decrease in the size of the spleen and the enlargement of the spleens in the majority of the controls as a result of the haematopoiesis revealed by microscopic examination. However, in gerbils with microscopically normal spleens (i.e. similar to those of other normal rodents such as rats and mice), body and spleen weights of males fed the 1% diet were almost identical to those of the controls, and those of females were only slightly lower. The values for these histologically normal gerbil spleens in the control and 1% diet groups are probably within normal limits. Furthermore, they are only slightly below the values reported as normal by Kramer (1964). Thus, spleens that can probably be regarded as normal in size are those weighing from 68 to 108 mg (0·75–1·10 g/kg body weight) and measuring from 1·3 × 0·4 × 0·3 cm to 1·8 × 0·6 × 0·5 cm in adult males weighing from 78 to 115 g, and those weighing from 62 to 100 mg (0·70–1·10 g/kg) and measuring from 1·0 × 0·5 × 0·2 cm to 1·8 × 0·5 × 0·5 cm in adult females weighing from 70 to 105 g. As in rats and mice, a spleen longer than the distance from the inner canthus of the eye to the tip of the snout is considered to be enlarged. Splenomegaly, by all these criteria, was present in 20 male and 17 female controls (slight in 14, moderate in 13 and severe in 10), four males and three females fed the 1% diet (slight in three, moderate in two and severe in two), one female fed 2% (slight) and one male fed 4% (slight). Microscopic examination of the gerbils in the dietary study revealed a positive correlation between gross splenomegaly and haematopoiesis; both were significantly greater in the controls (averaging slight to moderate) than in any of the three treated groups (minimal to very slight).

Spleen pathology in the intubation study

Although the spleens of many animals were the site of haematopoiesis and occasional ones showed acute splenitis or focal granulomas, none of these lesions bore any relation to dose.

Cardiac pathology

In the dietary study there was less myocardial fibrosis in the treated groups than in the controls and an inverse relation to dose was even suggested, but

Table 8. *Tumour incidence in gerbils fed erythosine in the diet for 24 months*

	Number of gerbils							
	Males				Females			
Type of tumour — Dose level (%)...	0	1	2	4	0	1	2	4
Hepatic cell adenoma, liver	1	0	0	0	0	0	0	0
Pheochromocytoma, adrenal	0	0	0	0	1	0	0	0
Cortical adenoma, adrenal	0	0	0	0	0	1	0	0
Granulosa-cell tumour, ovary	—	—	—	—	5	2	0	0
Granulosa-cell carcinoma, ovary*	—	—	—	—	0	1	0	0
Serous cystadenoma, ovary	—	—	—	—	1	0	0	0
Seromucous cystadenoma, ovary	—	—	—	—	0	0	0	1
Adenocarcinoma, intestine*	0	0	0	1	0	1	0	1
Papillary adenoma, intestine	0	0	0	0	0	0	0	1
Islet-cell adenoma, pancreas	0	0	0	0	1	0	0	0
Islet-cell carcinoma, pancreas*	0	0	0	0	0	1	0	0
Vascular hamartoma, kidney	0	1	0	0	2	0	1	0
Adenocarcinoma, kidney*	0	1	0	0	0	0	0	0
Transitional-cell papilloma, bladder	0	2	0	0	0	0	0	0
Total no. of tumours...	1	4	0	1	10	6	1	3
No. of gerbils:								
with tumours...	1	3	0	1	9	4	1	3
with malignant tumours...	0	1	0	1	0	3	0	1
started on experiment...	32	15	16	15	32	16	15	16
examined...	30	15	15	14	29	15	13	15

*Malignant tumour.

the amount of this lesion in both the controls and the treated groups was too small to ascribe the apparent decrease to erythrosine. Minimal degrees of myocardial necrosis were also found in 19 hearts, but the percentage of gerbils affected was approximately the same in each of these four groups and the lesion could not be attributed to erythrosine. In the intubation study there was no evidence of an effect upon the heart.

Other non-neoplastic lesions unrelated to erythrosine

Chronic interstitial nephritis, chronic glomerular nephritis, calcification of the tracheal cartilages, simple follicular cysts of the ovary, subacute to chronic endometritis and cystic endometrial hyperplasia were seen in approximately one third to three quarters of the gerbils in the dietary study. Their incidence did not differ significantly among the four groups and none of these lesions could be attributed to erythrosine. A small number of granulomas similar to those in the liver were also found in the mesenteric lymph nodes, spleen, bone marrow, lung, oesophagus, stomach and salivary glands from a few animals from each dietary level. Other non-neoplastic lesions were occasionally encountered in each group; none could be ascribed to erythrosine. Pneumonia, which is common in the ageing rat, was seen in only two gerbils. In the intubation study, similar lesions were found in approximately equal incidence in both treated and control groups; none could therefore be attributed to erythrosine administration.

Tumours in the dietary study

There was no evidence of any effect of erythrosine on tumorigenesis, as the incidence either of total tumours or of any single type was not significantly different among the four dosage groups (Table 8). The greater number in females was due primarily to the relatively high incidence of the benign ovarian granulosa-cell tumour, the most common neoplasm. The second most common tumours were vascular hamartoma of the kidney (sometimes called 'haemangioma') and mucinous adenocarcinoma of the intestine. Multiple tumours occurred in three gerbils: a control female had renal vascular hamartoma and ovarian serous cystadenoma, a male fed the 1% diet had renal vascular hamartoma and transitional-cell papilloma of the urinary bladder and a female fed the 1% diet had adrenal cortical adenoma, ovarian granulosa-cell tumour and mucinous adenocarcinoma of the colon.

Tumours in the intubation study

Table 9 shows that, as in the dietary study, there was no evidence of an effect of erythrosine on tumorigenesis, since there was no real difference in the incidence of total tumours or of any single type among the four groups. Tumour incidence approximated to the relatively low percentage noted in the dietary experiment, and similar types were encountered in both. Neoplasms were found in only four of the 98 males started but in 15 of the 98 females. As in the feeding study, the greater number in females was largely due to the relatively high incidence of ovarian tumours.

DISCUSSION

It is axiomatic that an effect of treatment can be substantiated only if normal controls are available for comparison. As we had had no previous pathological experience at the FDA with the Mongolian gerbil, *Meriones unguiculatus*, and general experience with this small rodent as a laboratory animal has also been limited, the problem of ascertaining whether the dietary administration of erythrosine had induced

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Table 9. *Tumour incidence in gerbils given erythrosine by intubation twice weekly for 22 months*

Type of tumour	Dose level (mg/kg)...	Number of gerbils							
		Males				Females			
		0	200	750	900	0	200	750	900
Islet-cell adenoma, pancreas		1	0	0	0	1	0	0	2
Luteal-cell adenoma, ovary		—	—	—	—	2	0	2	0
Leiomyoma, ovary		—	—	—	—	1	0	0	0
Granulosa-cell tumour, ovary		—	—	—	—	1	0	1	0
Granulosa-cell carcinoma, ovary*		—	—		—	0	2	0	0
Haemangioma, kidney		0	0	1	1	1	0	0	1
Cortical adenoma, kidney		0	0	0	0	0	0	0	1
Cortical adenoma, adrenal		0	0	0	0	0	0	0	1
Adenocarcinoma, small intestine*		0	0	1	0	0	0	0	0
Total no. of tumours...		1	0	2	1	6	2	3	5
No. of gerbils:									
with tumours...		1	0	2	1	6	2	3	4
with malignant tumours...		0	0	1	0	0	2	0	0
started on experiment...		33	20	22	23	30	20	22	26
examined...		30	18	20	22	27	19	22	26

*Malignant tumour.

changes in this animal was compounded by our finding of granulomas in the livers of the controls. Several questions were thus raised. What was the aetiology of the granulomas? If the livers of the controls were abnormal, was there any way to detect a possible effect on this organ in the treated groups, might not other organs likewise be abnormal and how could any possible effects on other organs also be proved?

Several theories have been proposed to explain the aetiology of the granulomas in the livers of the controls. The most common cause of this type of reaction in the vertebrate is probably tuberculosis; others include other mycobacterial infections such as leprosy, certain fungal infections, brucellosis, tularaemia, animal parasites, foreign bodies such as talc and starch, certain minerals such as beryllium and silicon, and, in man, syphilis and other treponemal infections, lymphogranuloma venereum, cat-scratch disease and Boeck's sarcoid (Allison, 1971; Cameron, 1962; Puckett, 1971; Schwarz, 1971). Certain triphenylmethane dyes, such as gentian violet, methyl violet, malachite green, brilliant green and fuchsin (rosaniline) have been shown to have bactericidal and fungicidal properties against Gram-positive (but not Gram-negative) micro-organisms (Grollman & Grollman, 1970). Erythrosine, of course, is not a triphenylmethane derivative but a tetraiodofluorescein. Thus, any antimicrobial effect it may possess is probably due to its iodine content, as iodine and iodides are known to be strong cutaneous antiseptics and to be effective also against internal infections caused by syphilis, amoebiasis and such fungal diseases as actinomycosis, oidomycosis, blastomycosis and sporotrichosis. There are some data to suggest an antituberculous action, also. However, these compounds are generally felt to be contraindicated in tuberculosis; some studies have shown that they may convert dormant into active disease and that their primary mode of action is not against micro-organisms *per se* but against the devitalized tissue characteristic of most granulomatous infections, with consequent release of organisms and exposure to immune substances and true anti-infective agents (Goodman & Gilman, 1970; Sollman, 1957). An antimicrobial role has not yet been proved for erythrosine, but the decrease in the number and size of the granulomas with increasing dose and their presence in other organs of a small number of gerbils, also in the dietary experiment, suggest that they were induced by a micro-organism which was destroyed by the red dye. Although the granulomatosis was more severe in the livers of the controls than in those of the treated gerbils in the intubation study, the dose effect observed in the dietary study was absent; this absence may be explained by the lower dosages, since the highest level by intubation (900 mg/kg) was slightly below the lowest administered by diet (1%) and the difference between animals fed the 1% diet and controls was not very striking. The failure to detect any bacteria or other micro-organisms by the special stains does not rule out their presence since no fresh tissue remained for preparing cultures, which are sometimes successful in identifying such organisms when simple staining fails.

The negative reactions to the special stains just mentioned and the presence in many livers of a pigmented lipid, identifiable as ceroid, led to a second theory to account for the hepatic granulomas (F. B. Johnson, Armed Forces Institute of Pathology, Washington, D.C., personal communication, 1969). It was suggested that they might be the result of deposition of ceroid secondary to either an excess of unsaturated fat or a deficiency of vitamin E or other antioxidant in the diet. Deposition of ceroid has been induced in experimental animals by such dietary manipulation (Hartroft & Porta, 1965). In addition, Gordon *et al.* (1961) induced granulomas in the gerbil liver by feeding the animals 1% cholesteryl methyl ether, which inhibits the absorption of cholesterol in the rabbit (Gordon & Cekleniak, 1959), showing that interference with lipid metabolism can be an aetiological factor in this animal. The major objection to the ceroid theory is that the pigment did not show the relation to dose which was characteristic of the granulomas, there being approximately equal pigmentation at all

four dose levels. Furthermore, it was demonstrable in only a small number of granulomas, most being concentrated within macrophages not located within granulomas but occurring either singly or in clusters. Therefore, a relative dietary deficiency of antioxidants may have been responsible for the intrahepatic deposition of ceroid, but relevance to the formation of the hepatic granulomas has not been proved.

A third possible source of the granulomatosis was proposed (H. L. Richardson, FDA, personal communication, 1970). It was suggested that this type of sarcoid-like reaction was highly suggestive of an autoimmune disease or a sensitization to an ingredient in the control diet. This theory does not rule out a microbial aetiology, as the type of granuloma seen in these gerbils is characteristic not only of tuberculosis and the other infectious diseases already mentioned but also of delayed or cell-mediated hypersensitivity, which is now known to be a factor in the development of these and other infections caused by parasites, viruses, Mycobacteria and certain other bacteria (Fenner, 1965; Medawar, 1959; Rhodes & Rozee, 1965; Waksman & Morrison, 1951).

While the aetiology of the granulomatosis remains unsolved, the presence of this lesion in the controls did not prevent the detection of an effect of the colouring on the liver. Although the mechanism involved remains obscure, the feeding of erythrosine decreased the degree of granulomatosis in this organ. There was also a slight increase in Kupffer-cell proliferation, but it appears that the colouring was more beneficial than harmful, as overall hepatic pathology was less in animals fed the 4 and 2% diets than in those fed the 1% diet and in the controls.

Even though the livers of control animals were abnormal, the effects of erythrosine on this organ could be detected. By comparing the weight, relative weight (ratio of organ weight to body weight), anatomical measurements and gross and microscopic appearance of the various organs of the gerbils in this study with those of similar rodents (rats and mice) and other colonies of gerbils (Kramer, 1964; Wilber & Gilchrist, 1965), it was possible to differentiate the normal from the abnormal with reasonable certainty. We were thus able to divide the lesions in the gerbils into three groups: those apparently effected by erythrosine; those which were probably unrelated to this food colouring but in which the incidence was significantly different in treated and control animals and those of approximately equal incidence in treated and control groups and therefore unrelated to the feeding of erythrosine.

Organs, in addition to the liver, that were affected by erythrosine in the dietary study were the thyroid, the spleen and possibly the heart. In contrast to the liver, the thyroids in the control gerbils were free of granulomas, were all of essentially similar appearance, and their gross and microscopic structures were like those of the mouse and rat thyroids. However, changes resembling those of human nodular (adenomatous) goitre were seen in thyroids of animals treated at the high level. This is the most common disease of the thyroid in man, and is basically due to the inability of the gland at certain periods to form sufficient thyroxin (T_4) with four atoms of iodine and triiodothyronine (T_3) with three, or, in a small percentage of cases, to release the hormones into the blood. The chief cause is dietary deficiency of iodine, although paradoxically an excess of iodine can produce the same effect (Grollman & Grollman, 1970; Hazard, 1971; Sommers, 1971).

It is now generally conceded that nodular goitre follows repeated cycles in which in the early stage, rarely seen by the pathologist, there is hyperplasia followed by regession of the follicular epithelium to the squamous state with increased storage of hormone as colloid when the impediment to synthesis is removed. These cycles lead to an increase in size of individual follicles and the whole gland, with the storage phase eventually becoming predominant. Compression of more normal areas and their blood supply by expanding areas leads to necrosis, haemorrhage, deposition of cholesterol crystals, infiltration of macrophages and foreign body giant cells, and subsequent focal fibrosis, calcification and nodularity (Follis, 1959; Hazard, 1971; Marine and Williams, 1908; Sommers, 1971).

The similarity of this picture to the changes observed in the thyroids of the gerbils treated by dietary administration of erythrosine is evident. As in human nodular goitre, the most marked alteration in the gerbils was enlargement of the majority of the follicles associated with a simple squamous epithelial lining and distension of the lumina with colloid, indicating increased storage of thyroglobulin. Also as in man, foci of small follicles were seen consistently. However, in the stage of the human disease usually seen by the pathologist, the small follicles tend to resemble the large ones except for size, whereas in the gerbil many appeared to be hyperplastic because of their cuboidal to columnar epithelium and sparse colloid. Total hyperplasia indicated by this change and by intraluminal invagination of papillae covered by proliferating columnar epithelium was found in a total of 19 treated gerbils, but in none of the controls, and increased with increasing dose. Another feature observed in these animals and often seen in the human disease was intraluminal and interstitial leucocytic infiltration, indicating thyroiditis, but it was not as clearly related to dose as the other changes. Follis (1964) reported pronounced thyroiditis in the hyperplastic glands of hamsters given large doses of potassium iodide. Characteristics of human nodular goitre not found in these animals are haemorrhage, necrosis, giant cells, cholesterol crystals, fibrosis, calcification, and true nodularity. Human patients with this disease are usually euthyroid, occasionally hyperthyroid (if hyperplasia is marked), and only rarely (largely in cretinous infants) hypothyroid. Carcinogenesis is rarely, if ever, associated with nodular goitre in man (Sommers, 1971), and none of the thyroids in the gerbils in this study contained either benign or malignant tumours.

Erythrosine administered by intubation did not appear to induce any effects in the thyroid or any other organ. The absence of any clear effect on the thyroid may be explained by the lower dosage levels and the less frequent administration of the compound.

Changes in the thyroid have not been reported in any of the numerous studies of erythrosine in the rat, including seven experiments lasting between 1·5 and 2 yr (Hansen *et al.* 1973a,b; Umeda, 1956; Willheim

& Ivy, 1953). A 2-yr study by the FDA in which levels ranging from 0·5 to 2% were fed to dogs also failed to produce alterations in this organ (Hansen *et al.* 1973a). Not surprisingly, in view of the iodine in the compound, the protein-bound iodine has been reported by several investigators to be elevated in the rat as well as in man following administration of erythrosine (Andersen, Keiding & Neilsen, 1964; Bowie *et al.* 1966; Hansen *et al.* 1973b). It was not measured in the gerbil in either the feeding or the intubation study because of difficulties in obtaining blood.

The decrease in haematopoiesis observed in the spleen is a common reaction to necrosis or severe inflammation in rodents. It ranged from the slight to moderate degree in the controls to the minimal amount which may be regarded as within normal limits in the treated animals and appeared to be related to the decrease in hepatic granulomatosis. The mild reduction in haematological values in the treated animals in the dietary study was probably an effect of inanition, since the bone marrow appeared to be of normal cellularity, loss of weight occurred in all three groups of treated animals and reticulocytosis was absent. The significance of the reduction in erythrocytes without associated changes in other haematological parameters in the treated male gerbils at 12 and 22 months in the intubation study is not clear. Erythrosine-induced anaemia was also reported to occur in rats given intubations of the colouring for 3 months (Bowie *et al.* 1966). However, no effect upon the haematopoietic system of the rat was noted either by Hansen *et al.* (1973b) after oral intubation and feeding for 85 and 86 wk, respectively, or by Hansen *et al.* (1973a) after dietary administration for 2 yr; the latter group also failed to observe any effect of this nature in dogs fed with erythrosine for 2 yr. Haemolytic anaemia in mice was reported by Waliszewski (1952), but there is no evidence that the mild anaemia in these gerbils was attributable to haemolysis. An increase in the coagulating ability of the blood has been suggested by certain experiments in the past. Rapid clotting was noted in rats fed levels ranging from 0·5 to 5% for 2 yr (R. E. Zwickey, FDA, personal communication 1956), and Bowie *et al.* (1966) reported that rats treated for 6 months had shortened clotting and prothrombin times. There was no evidence of altered coagulating ability in the gerbils. Although this condition is often associated with vascular disease, infarction and fibrosis, there has been no indication of an increase in any of these lesions in any of the erythrosine studies so far performed in animals. In fact, in the gerbils treated by dietary administration, there was evidence suggesting that there may have been less myocardial fibrosis in the treated groups than in the control.

Mesenteric lymph-node disease exemplifies the second category of lesions, i.e. those that were probably unrelated to the administration of the colouring but in which the incidence was significantly different in treated and control groups. It was three times as severe in the control males as it was in any other group. However, aside from this, no evidence of a correlation with dosage or with any other abnormality was apparent and there is no reason to associate the decrease with erythrosine. It was almost identical to the disease in the mouse, in which it is also common in untreated animals. The aetiology is not known in either species.

Lesions in the third category, i.e. those of approximately equal incidence in both treated and control groups and thus unrelated to the feeding of erythrosine, have already been discussed. Included in this class are tumours. As none of the many studies on this food colouring in a variety of species has shown an increase in either malignant or benign tumours, it is probably safe to conclude that erythrosine is not a carcinogen or tumorigen.

Except for the liver, thyroid and haematopoietic system, which have been discussed, there was no evidence of an effect of erythrosine on the organs of the gerbils treated by intubation.

The Mongolian gerbil, *Meriones unguiculatus*, is a relatively new laboratory mammal. Data on this animal have been collected by various investigators, but more detailed studies are needed before its anatomical, physiological, behavioural, biochemical, haematological, pathological and bacteriological norms can be established. In particular, the most serious problem encountered in this experiment, the aetiology of the hepatic granulomas, which were so prominent in the controls, requires a solution. Other investigators (Clarkson *et al.* 1957; Gordon and Cekleniak, 1960; Gordon *et al.* 1961), stating that the gerbil readily develops hypercholesterolaemia but not atherosclerosis after dietary exposure to only moderate amounts of fat and/or cholesterol, have commented on its value in research on atherosclerosis. Our study has shown that, if granulomatosis can be eliminated, the gerbil should also compare favourably with the rat and mouse as a valuable rodent for chronic toxicity and carcinogenicity testing in general, because of its ease in handling, virtual freedom from pneumonia, clear response to dose and low spontaneous tumour incidence in general combined with some tendency to develop intestinal adenocarcinoma, which is a neoplasm rare in most strains of rats and mice but the second most common cancer in man (Burkitt, 1975). If an effect on the thyroid is suspected, the gerbil may actually prove superior to the rat and dog. Erythrosine in high doses has been tested for long periods in these species as well as in the gerbil, and, for reasons already discussed, this colouring would be expected to induce thyroid lesions because of its high iodine content. Only in the gerbil have such lesions been demonstrated.

Conclusions

Administration of erythrosine to gerbils at levels of 1, 2 or 4% in the diet for 2 yr produced a dose-related change in the thyroid reminiscent of human nodular goitre. Other deleterious effects of treatment were slight depression of haematological values and dose-related loss of weight at levels of administration of 1% and higher. The colouring also induced a dose-related decrease in the incidence and severity of granulomatosis, a disease of undetermined aetiology in the livers of the controls. Survival of animals fed 1 and 2% erythrosine diets was greater than that of controls. In gerbils intubated with erythrosine, some decrease in granulomatosis was evident at the lowest

level tested, 200 mg/kg, and there was no deleterious effect in gerbils given doses below 750 mg/kg. As there is evidence that the granulomas may have been of microbial aetiology, further investigation of erythrosine as a possible antimicrobial agent is warranted.

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SHORT-TERM TOXICITY OF HYDRATROPIC ALDEHYDE IN RATS

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Abstract—Hydratropic aldehyde, dissolved in corn oil, was given to rats by oral intubation at dose levels of 0 (control), 10, 50 or 500 mg/kg body weight/day for 15 wk. Differences between treated and control groups in the mean rate of body-weight gain, in food consumption and in renal function tests were not statistically significant. There were statistically significant increases in water consumption at the highest dose level and small reductions in haemoglobin concentration in rats given 50 or 500 mg/kg/day. The liver weight was increased in animals receiving 500 mg/kg/day, without any histopathological change. Slight increases in relative liver weight were noted with 10 and 50 mg/kg/day, but these were not considered to be related to treatment. Increased weights at 500 mg/kg/day were seen in the kidney, stomach and pituitary gland in both sexes and, in females alone, in the heart and spleen. The histological appearance of all tissues was similar in the test and control groups. The no-untoward-effect level was 10 mg/kg/day, which is more than 100 times the estimated maximum intake by man.

INTRODUCTION

Hydratropic aldehyde (2-phenylpropanal) is used as a constituent of flavourings in sugar and flour confectionery, ice-cream and soft drinks.

At present there are no specific regulations governing the use of flavouring agents in the UK. The Food Standards Committee (1965), in its Report on Flavouring Agents, considered representations concerning hydratropic aldehyde, but it was not listed among those materials recommended to be prohibited for use in food. It is classified by the Council of Europe (1972) as a permissible additive to foodstuffs up to a limit of 1 ppm and is permitted in the USA under Section 121.1164 of the Code of Federal Regulations.

The LD_{50} value after oral administration in the rat is 2·8 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). Metabolic studies have been confined to rabbits, in which it was found that approximately half of an oral dose of hydratropic aldehyde was excreted as an ester glucuronide (Robinson, Smith & Williams, 1955). There are no data available on the safety-in-use of hydratropic aldehyde. The present paper describes a short-term toxicity study carried out as part of a programme of safety evaluation of flavouring agents.

EXPERIMENTAL

Materials. The sample of hydratropic aldehyde used was supplied by Firmenich & Co., Geneva, Switzerland, and complied with the following specification: Purity, min. 98%; specific gravity (at 20°C), 1·001–1·009; refractive index, 1·516–1·519; acid number, max 5·0. Corn oil was supplied by J. Sainsbury Ltd.

Animals and diet. Rats of the CFE strain and of both sexes were obtained from a specified-pathogen-free breeding colony and given Spillers' Laboratory Small Animal Diet and water *ad lib.* They were housed five in a cage in an animal room maintained at 21 ± 1°C and 50–60% relative humidity.

Loss of hydratropic aldehyde from animal diet. A diet containing 5% hydratropic aldehyde was prepared and exposed to the air in the animal room for 18 hr. The loss in weight of the diet, corrected for the loss in weight of a control sample without the aldehyde, showed that 35% of the aldehyde was lost in this time. In view of its volatility and low solubility in water the flavouring could not be administered to the rats in the diet or drinking-water and was given instead by daily oral intubation as a solution in corn oil.

Experimental design. Hydratropic aldehyde was given by daily oral intubation (7 days/wk) to groups of 15 male rats (body weight 65–100 g) and 15 females (body weight 60–90 g) for 15 wk at dose levels of 0 (control), 10, 50 and 500 mg/kg body weight/day. In addition, groups of five rats of each sex were given daily doses at the same levels for 6 wk and groups of ten male and five female rats were treated for 2 wk. The concentrations of the solutions were selected so that each rat was given 5 ml/kg of the appropriate solution, the controls being given corn oil.

The rats were weighed weekly up to wk 14 and the consumption of food and water was determined during the 24 hr preceding each weighing. On the day after the final dose, the animals were deprived of food for 24 hr and killed by exsanguination from the aorta under barbiturate anaesthesia and the blood

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was used for haematological examinations and serum analyses. The blood was examined for haemoglobin content, packed cell volume and counts of erythrocytes and leucocytes. Slides were prepared from all blood samples for counting of reticulocytes and the different types of leucocytes but these counts were confined, in the first instance, to the controls and animals on the highest level of treatment. Reticulocytes were also counted in the samples from the rats given 50 mg/kg/day for 15 wk. The serum was analysed for urea, glucose, total protein and albumin as well as for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

At autopsy all the animals were examined for gross abnormalities and the brain, pituitary, thyroid, heart, liver, spleen, kidneys, adrenal glands, gonads, stomach, small intestine and caecum were weighed. Samples of these organs and of lung, salivary gland, aorta, thymus, various lymph nodes, urinary bladder, colon, rectum, pancreas, uterus, spinal cord and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination.

Urine was collected during the final week of treatment and examined for appearance, microscopic constituents and content of albumin, glucose, ketones, bile salts and blood. Renal concentrating and diluting ability was assessed at the same intervals by measuring the specific gravity and volume of the urine produced during a 6-hr period of water deprivation and in the 2-hr period after a water load of 25 ml/kg. In addition, at wk 6 and 14, the same measurements were made on the urine produced during a 4-hr period commencing after 16 hr without water. The number of cells in the urine was counted using the 2-hr sample.

RESULTS

No deaths and no abnormalities in behaviour were seen during the study. The appearance of the rats was normal throughout, except for one male control rat in which a subcutaneous swelling in the neck was seen at wk 6. At post-mortem examination at wk 15, this swelling was found to be a fluid-filled cyst, 5 cm in diameter, attached to a salivary gland and histopathological examination showed it to be a cyst of the mixed submandibular salivary gland.

Although the males given the two highest levels of hydratropic aldehyde gained slightly less weight than the controls, the differences were not statistically significant (Table 1). No differences between treated and control groups were detected in the male rats given 10 mg/kg/day or in the females at any dose level. However, at the highest dose level the overall food intake was slightly increased in both sexes, whilst the overall water intake in the same group was increased by 15 and 21%, compared with the controls, in males and females respectively. The difference in the females was statistically significant, as were approximately half the weekly values, particularly in the latter half of the study. In the males the differences in water intake between treated and control groups were statistically significant only at 28, 41 and 56 days.

The haemoglobin concentration was decreased by 5–7% compared with the controls in the males given 500 mg hydratropic aldehyde/kg/day at wk 6 and in both sexes at wk 15 (Table 2). It was decreased by a similar amount in the females given 50 mg/kg/day for 15 wk. Compared with the controls there was a slight polycythaemia in males given 50 mg/kg/day for 15 wk but there were no similar findings at the higher dose level or in females. The reticulocyte count was increased at wk 15 in females given 500 mg hydratropic aldehyde/kg/day but that in rats given 50 mg/kg/day was normal. The results of the serum analyses were similar in test and control rats.

The urine of all the rats was free from bile, blood, glucose and ketones and the concentrations of albumin were similar in all groups. The renal concentration and dilution tests showed no statistically signi-

Table 1. *Body weights and food and water consumption of rats given daily doses of 0–500 mg hydratropic aldehyde/kg for up to 14 wk*

Dose level (mg/kg/day)	Body weight (g) at wk 0†	1	5	9	14	Mean food consumption (g/rat/day)	Mean water consumption (ml/rat/day)
Males							
0	86	137	340	448	526	18·7	27·6
10	84	135	333	435	532	18·9	26·6
50	86	138	318	418	489	18·0	25·8
500	87	138	323	424	482	20·3	31·7*
Females							
0	78	120	224	272	301	15·0	23·3
10	78	124	223	271	303	15·8	22·9
50	78	120	222	270	302	15·7	23·2
500	78	118	222	271	303	16·0	28·2**

†First day of dosing.

Body-weight values are means for groups of 15 animals. Values for food and water consumption are based on mean data for three cages of five animals, and those marked with asterisks differ significantly (White, 1952) from the corresponding control figure: $*P < 0·05$; $**P < 0·01$.

Table 2. *Haematological values in rats given daily doses of 0–500 mg hydratropic aldehyde/kg for 15 wk*

Dose level (mg/kg/day)	No. of rats	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	L	M
				Males						
0	15	14·5	45	6·7	0·7	5·6	13	1	85	1
10	15	14·6	45	7·1	—	5·1	—	—	—	—
50	15	14·5	45	7·2*	0·8	5·4	—	—	—	—
500	15	13·7*	43	6·9	0·7	4·9	17	1	81	1
				Females						
0	14	14·3	43	6·1	0·5	4·1	14	1	84	1
10	15	13·9	43	5·9	—	3·7	—	—	—	—
50	14	13·8*	43	5·8	0·4	3·7	—	—	—	—
500	15	13·7*	42	6·1	0·8*	4·8	13	1	85	1

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

Figures are means for the numbers of rats shown, and those marked with an asterisk differ significantly from the corresponding control figure: $*P < 0{\cdot}05$ (Student's t test).

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

ficant differences between treated and control animals, nor were there any differences in cell excretion rates.

At autopsy, small lung lesions were observed, and histopathological examination revealed pulmonary leucocyte infiltration, suggesting a mild respiratory infection.

Most of the differences between treated and control rats in the organ weights and organ weights expressed relative to body weight (Table 3) were confined to the rats given 500 mg hydratropic aldehyde/kg/day. The most consistent of these was an elevated liver weight present in both sexes at all three examinations. In addition, the relative liver weights were increased at the intermediate dose level (50 mg/kg) in the males after 2 and 15 wk as well as in the females after 6 wk. The only similar change at the lowest dose level (10 mg/kg/day) occurred in the males at wk 15.

In the case of the kidney and the stomach, the values for relative organ weight were increased at wk 6 and 15 at the highest dose level. In the same animals the stomach weights were increased to a statistically significant degree only in females at wk 15, whereas the kidney weights were significantly higher than the controls in all except the females at wk 6.

The remaining, more isolated, changes in comparison with the controls were a lower relative brain weight and a higher spleen weight in females given 10 mg/kg/day at wk 6, an increased relative spleen weight in females given 500 mg/kg/day at wk 15, an increased relative small intestine weight in males given 500 mg/kg/day at wk 6, increased relative pituitary weights in males given 500 mg/kg/day at wk 6 and 15 and in females on the same level at wk 2, a decreased relative ovary weight at 50 mg/kg/day after 6 wk and an increased thyroid weight in males given 500 mg/kg/day at wk 2. The heart and relative heart weights were higher than the control values in the females given the highest dose level for 15 wk. A similar change was seen in the relative heart weight of males on the highest and the lowest dose levels at wk 6.

On microscopic examination of the tissues, a small granuloma was seen in the liver of one male rat after treatment for 15 wk with 500 mg hydratropic aldehyde/kg/day. No histological changes related to the period or level of treatment were seen in any of the organs examined.

DISCUSSION

The increased intake of water by rats on the highest dose level of hydratropic aldehyde is in keeping with previous observations in our laboratories that rats given high concentrations of flavouring agents by intubation tend to drink increased quantities of water (Brantom, Gaunt, Grasso, Lansdown & Gangolli, 1972; Colley, Gaunt, Lansdown, Grasso & Gangolli, 1969; Gaunt, Agrelo, Colley, Lansdown & Grasso, 1971; Gaunt, Mason, Hardy, Lansdown & Gangolli, 1974). The reason for these increases, sometimes accompanied by increases in food intake, are unknown. However, it has been suggested (Gaunt *et al.* 1974) that dosing with highly flavoured solutions during the day, when rats do not normally eat or drink, may stimulate the animals to consume small quantities of food or water.

Apart from this, the principal findings with hydratropic aldehyde were increases in the weights of several organs, notably the liver, kidney and stomach, in animals treated with the highest dose level of 500 mg/kg/day. There were also small haematological changes.

The increase in liver weight was not accompanied by evidence of pathological changes and it is probable that it was a result of the induction of microsomal processing enzyme activity by the large amounts of hydratropic aldehyde administered. Robinson *et al.* (1955) found that rabbits dosed orally with hydratropic aldehyde excreted 51–54% of the dose as glucuronide, and they succeeded in isolating hydratropoylglucuronide from the urine. This was identical to a conjugate obtained after administration of hydratropic acid or isopropylbenzene (cumene). The absence

Table 3. *Relative organ weights of rats given daily doses of 0–500 mg hydratropic aldehyde/kg for 2, 6 or 15 wk*

Sex and dose level (mg/kg/day)	No. of rats	Relative organ weights (g/100 g) Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	Terminal body weight (g)
Wk 2														
Male														
0	10	0·99	0·43	3·49	0·43	0·82	0·66	4·05	0·49	29·0	1·17	3·8	7·9	158
10	10	1·01	0·45	3·59	0·41	0·87	0·67	4·30	0·46	27·3	1·13	4·2	7·4	171
50	10	1·07	0·45	3·75*	0·44	0·89*	0·67	4·20	0·56	30·4	1·12	4·5	7·6	164
500	10	1·00	0·42	4·32**	0·43	0·88	0·71	4·59	0·50	30·5	1·15	3·5	8·9	162
Female														
0	5	1·17	0·48	3·71	0·48	0·88	0·75	4·58	0·51	40·7	79·3	5·9	8·8	126
10	5	1·12	0·44	3·87	0·46	0·81	0·74	4·51	0·51	36·9	74·5	5·7	10·4	132
50	5	1·15	0·43	3·83	0·46	0·90	0·76	4·73	0·54	41·3	81·4	6·3	11·0	134
500	5	1·18	0·46	4·46***	0·41	0·95	0·77	4·37	0·52	40·9	83·0	6·5*	11·0	126
Wk 6														
Male														
0	5	0·53	0·31	2·86	0·24	0·65	0·48	2·39	0·29	18·5	1·03	2·7	6·9	338
10	5	0·56	0·34*	3·02	0·24	0·73	0·52	2·53	0·33	18·1	1·05	2·8	6·1	314
50	5	0·56	0·33	2·97	0·26	0·72	0·48	2·52	0·31	17·7	1·16	3·0	6·6	329
500	5	0·58	0·35*	3·88**	0·26	0·82**	0·56*	2·72*	0·32	21·0	1·11	3·3**	7·1	312
Female														
0	5	0·82	0·39	2·98	0·27	0·72	0·60	3·36	0·40	39·4	70·1	4·1	9·3	204
10	5	0·73*	0·37	3·08	0·30	0·75	0·60	3·35	0·37	34·8	59·6	4·3	7·3	225
50	5	0·80	0·38	3·14*	0·30	0·72	0·65	3·20	0·37	34·1	52·8	4·6	7·7	216
500	5	0·80	0·42	4·46***	0·31	0·83	0·72*	3·35	0·40	39·4	59·5	5·0	6·4	203
Wk 15														
Male														
0	15	0·37	0·27	2·36	0·17	0·54	0·39	1·72	0·24	11·7	0·77	2·3	4·3	507
10	15	0·39	0·28	2·57**	0·18	0·58	0·40	1·81	0·23	12·2	0·79	2·4	4·7	493
50	15	0·40	0·28	2·53*	0·18	0·57	0·39	1·84	0·24	12·6	0·80	2·4	4·6	463
500	15	0·41	0·29	3·23***	0·19	0·70***	0·44**	1·89	0·26	13·5	0·82	2·7*	5·1	470
Female														
0	15	0·63	0·31	2·33	0·22	0·58	0·51	2·38	0·31	23·8	49·5	4·1	6·8	289
10	15	0·62	0·33	2·39	0·23	0·57	0·51	2·47	0·31	22·8	47·3	4·7	7·1	278
50	15	0·63	0·33	2·46	0·23	0·61	0·52	2·43	0·30	24·8	47·8	4·2	7·3	287
500	15	0·63	0·35**	3·68***	0·25	0·69***	0·58**	2·45	0·35	24·3	56·7	4·7	7·6	291

†Weights of this organ are expressed in mg/100 g body weight.
‡Weights of this organ in females are expressed in mg/100 g body weight.
Figures are means for the numbers of rats shown. Those marked with asterisks differ significantly (Student's *t* test) from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

of pathological changes in the present study suggests that the tissue concentration at which hepatotoxicity might have occurred had not been reached or exceeded at this rate of intake and that the liver enlargement did not represent an untoward effect.

Although there were increases in relative liver weight at the lower dose levels these were confined to the males at wk 15 and there was little evidence that the effect was dose-related. In the absence of any increase in the actual organ weights it is likely that these increased ratios were due to the lower body weights in these groups. This suggestion is supported by the observation that most of the relative organ weights in these groups were higher than the controls by 3–7% at the lowest dose and 4–8% in those rats given 50 mg/kg/day. The corresponding increases in relative liver weight were 9 and 7% respectively. Since the magnitude of the increases in liver weight was similar to that for the other organs, it seems likely that these all resulted from the lowered body weight, and the fact that the values for the liver were statistically significant may be fortuitous. Further evidence against these livers being enlarged is the fact that the values for the liver weights were closely similar to the values collected in our laboratories for normal animals in the same weight range. On the basis of these observations, it is unlikely that hydratropic aldehyde influenced the liver weight at these lower doses.

The increased kidney weights in both sexes after treatment for 6 and 15 wk were not accompanied by histopathological changes, nor were any functional defects detected. It is probable that the increased kidney weights were due, as with the liver, to some change in functional demand.

Administration of a 10% solution was necessary to achieve the highest dose level of hydratropic aldehyde and repeated exposure of the gastric mucosa to such a concentration could have been responsible for the increase in relative stomach weights recorded in both sexes at wk 6 and 15. There was, however, no autopsy or histopathological evidence of irritation or hyperplasia.

The increases in pituitary weight were not associated with histological changes in this or in the other endocrine glands. The reason for the increases is therefore not known, but in the absence of evidence to the contrary and since they were present in rats given the highest dose level, they must at present be considered to be related to hydratropic aldehyde treatment.

There were isolated changes in other organ weights after treatment at the 10 mg/kg/day level. For example the relative weights of brain and heart and the weight of the spleen were different from controls in one sex or the other after 6 wk. In all cases there was no significant change after prolonged treatment at this dose level, nor at 50 times the dosage. Therefore, these are considered to be fortuitous findings rather than a result of hydratropic aldehyde administration.

The polycythaemia in males given 50 mg/kg/day was not seen at the higher dose and was likely to be due to chance rather than to treatment. However, the decreases in haemoglobin concentration at the higher dosage levels after 6 and 15 wk were probably related to treatment. After 15 wk, the female rats showed evidence of greater susceptibility since, additionally, haemoglobin concentration was reduced at 50 mg/kg/day. The increase in reticulocyte count in females at 500 mg/kg/day suggests that erythropoiesis had been stimulated. These changes, together with the increased spleen weights, are consistent with an elevated red-cell turnover in this group. The reason for such an enhanced turnover rate is not known, but no inclusion bodies that might be responsible for a premature erythrophagocytosis were seen.

From the results of this study, it is concluded that the no-untoward-effect level for hydratropic aldehyde in the rat is 10 mg/kg/day. Bearing in mind that the dose was given once daily, a situation likely to lead to high blood levels, and that the changes observed at five times this dose were of a minor nature, it is likely that the true no-untoward-effect level may be nearer 50 mg/kg/day. Nevertheless, from data supplied by four leading flavouring manufacturers, it is calculated that the maximum likely intake of hydratropic aldehyde by man is between 2·0 and 4·4 mg/person/day or between 0·03 and 0·07 mg/kg/day for a 60-kg adult. Therefore the no-untoward-effect level from the present study is more than 100 times the estimated maximum intake by man.

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LONG-TERM TOXICITY OF CYCLOHEXYLAMINE HYDROCHLORIDE IN THE RAT

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Abstract—Groups of 48 rats of each sex were given diets containing 0, 600, 2000 or 6000 ppm cyclohexylamine hydrochloride (CHAH) for 2 yr. There was no indication of a carcinogenic effect at any of the levels of treatment. Findings that could be related to treatment were a slight anaemia, a failure to produce normally concentrated urine and an increase in the number of animals with lungs showing foamy macrophages within the alveoli among the rats given 6000 ppm CHAH. There were testicular changes in the form of atrophy or tubules with few spermatids in rats given 2000 or 6000 ppm CHAH. Decreased rates of body-weight gain, reduced food intakes, reduced water intakes, alterations of organ weights, lowered concentrations of serum urea, higher concentrations of serum albumin and reduced frequencies of many of the histopathological findings were observed in the treated rats compared with the controls. These differences were explicable in terms of changes resulting from a lowered body weight consequent on a decreased food intake. The conclusions from the study were that CHAH at levels up to 6000 ppm in the diet did not show any carcinogenic potential and that the no-untoward-effect level was 600 ppm.

INTRODUCTION

The uses of cyclohexylamine, together with data relating to its metabolism and toxicity, have been reviewed by Gaunt, Sharratt, Grasso, Lansdown & Gangolli (1974) and by Hardy, Gaunt, Hooson, Hendy & Butterworth (1976). These authors stressed that the current interest in this compound was due to its formation as a metabolite of the cyclamate sweeteners.

Gaunt *et al.* (1974) established a no-untoward-effect level of 600 ppm cyclohexylamine hydrochloride in the diet of rats as the result of a short-term study. At higher dietary levels (2000 and 6000 ppm) the rate of body-weight gain was reduced in association with a lower intake of an unpalatable diet. Paired feeding showed that limitation of food intake did not entirely account for the failure to gain weight at a normal rate. Since no abnormality of nutrient absorption was demonstrable, it was suggested that an increased metabolic rate might contribute to the reduced weight gain. Apart from a slight reduction in the renal concentrating ability of females on the highest dietary level, the most outstanding finding concerned the testes. There was a reduction in testicular weight and relative testicular weight, with histological evidence of reduced spermatogenesis, in the animals given 2000 or 6000 ppm cyclohexylamine hydrochloride.

Dietary levels of up to 3000 ppm cyclohexylamine hydrochloride did not influence the mortality, rate of body-weight gain, food intake, water intake, results of haematological examinations or the incidence of tumours in mice treated for 80 wk (Hardy *et al.* 1976). Similarly there was no effect on the incidence of the histopathological findings except for an increased incidence of minor hepatic changes in the females maintained on the diet containing 3000 ppm.

This paper reports the results of a long-term rat study designed to investigate the toxicity and carcinogenic potential of cyclohexylamine. The dose levels used were the same as those used in the earlier studies in rats (Gaunt *et al.* 1974) in order that the effects found in that experiment could be investigated after a longer period of exposure. The lowest of these levels was based on an estimate of the quantity of cyclohexylamine (approximately 30 mg/kg/day) that might be produced by total metabolism of cyclamate in an individual consuming 50 mg/kg/day. The level of 50 mg cyclamate/kg/day is the maximum daily intake suggested in the Eleventh Report of the Joint FAO/WHO Expert Committee on Food Additives (1968).

EXPERIMENTAL

Materials. The cyclohexylamine hydrochloride (CHAH) was prepared by Laporte Industries, Ilford, Essex, from cyclohexylamine conforming to the specification of the British Standards Institution (1968). It was from the same batch as that used in the previous short-term study in rats (Gaunt *et al.* 1974) and long-term study in mice (Hardy *et al.* 1976).

Animals and diet. Rats of both sexes of a Wistar strain obtained from a specified-pathogen-free colony were housed in a room maintained at 20 ± 1°C with a relative humidity of 50–60%. The basal diet, reground Spillers' Laboratory Small Animal Diet, and tap-water were provided *ad lib.*

Experimental design and conduct. Groups of 48 male rats (body weight 100–165 g) and 48 females (body weight 85–140 g) were housed four to a cage and given diet containing 0 (control), 600, 2000 or 6000 ppm CHAH. The rats were observed for signs of ill-health and any showing such signs were isolated. They were returned to the group cage if their condition improved, or killed and subjected to post-mortem

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examination if recovery was not obvious. The animals were weighed individually at the beginning of the experiment, at wk 1, 2 and 4 and then at 4-wk intervals. The quantities of food consumed by each cage of rats was measured at the same intervals, over the 24-hr period preceding the day of weighing, and water consumption was measured over the 48-hr period prior to the day of weighing.

During wk 14, 31, 52 and 80, blood samples were collected from a caudal vein of ten rats of each sex from the control group and those given 2000 or 6000 ppm CHAH and at wk 104 blood samples were collected at autopsy from the aorta of all surviving rats. At wk 80 the examination of the blood was confined to measuring the haemoglobin concentration and preparing a slide to identify the different types of leucocytes. In addition, at all other examinations, the packed cell volume was measured, a slide was prepared for counting reticulocytes and counts were made of the total erythrocytes and leucocytes. The counting of reticulocytes and the identification of the different types of leucocytes was confined to the slides from the control rats and those given 6000 ppm CHAH.

During wk 13, 26 and 52, urine samples were collected from ten control rats of each sex and from the same number given 6000 ppm CHAH. The urine was examined for the presence of protein, glucose, blood, bile salts and ketones. At the same intervals a concentration test was carried out involving measuring the volume and specific gravity of the urine produced during a 6-hr period of water deprivation, in the first 2 hr after a water load of 25 ml/kg and in a 4-hr period after 16 hr without water. A cell count was made using the urine collected for 2 hr after the water load.

Animals that died during the study were subjected to post-mortem examination unless this was precluded by advanced autolysis or cannibalism. All animals found to be *in extremis* during the study and those surviving at wk 104 were killed by an ip injection of barbiturate, bled from the aorta and examined for macroscopic abnormalities, while samples of brain, pituitary, thyroid, salivary glands, thymus, various lymph nodes, adrenal glands, pancreas, gonads, heart, aorta, lungs, trachea, oesophagus, stomach, small intestine, caecum, colon, rectum, liver, spleen, kidneys, skeletal muscle, spinal cord and uterus or prostate together with any other tissue that appeared to be abnormal were preserved in buffered formalin. The urinary bladder was fixed by filling, through the urethra, with Bouin's fixative and immersing in the same fixative. After 24 hr, the bladders were divided by a median sagittal section, examined with a low-power (× 10) microscope and transferred to 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for histopathological examination. During the post-mortem examination of the rats surviving to wk 104, the brain, heart, liver, spleen, kidneys, stomach, small intestine, caecum, adrenal glands, gonads, pituitary and thyroid were weighed.

RESULTS

The rats given the two higher dietary levels appeared healthier than the controls and those given the lowest level of CHAH, in that their coats remained relatively smooth and white instead of showing the yellowing and roughening usual in ageing rats. As is usual in a long-term study, there were deaths in all the groups (Table 1), but deaths were fewer in animals treated with CHAH than in controls, particularly at the higher levels of treatment (2000 and 6000 ppm). The differences in the numbers of animals dead was statistically significant in both sexes at the highest level of treatment, from wk 63 in females and from wk 87 in males. At the intermediate level (2000 ppm), significantly fewer females died, compared with the controls, from wk 75. At the lowest level (600 ppm) significant differences in death rate were found only occasionally, again in the females.

The behaviour of the rats given CHAH did not appear to differ from that of the controls but those given the highest dietary level (6000 ppm) were visibly smaller than those eating control diet. This was confirmed by the body-weight data (Table 2), which showed a dose-related reduction in the rate of body-weight gain throughout the study. Compared with the controls, males and females given 6000 ppm CHAH showed respectively a 31 and 41% reduction in body weight at wk 101. The corresponding figures in the rats given 2000 ppm CHAH were 14 and 24%, while at the lowest level of treatment (600 ppm) the reductions were 7 and 11%.

The food intakes (Table 3) of both sexes given 6000 ppm CHAH were reduced compared with the controls, the differences being statistically significant at most intervals throughout the study. The overall differences in consumption compared with the controls were approximately 10%. At the intermediate dietary level (2000 ppm) the corresponding reduction was approximately 5% and the individual values were less frequently reduced to a statistically significant degree. At the lowest level (600 ppm) there were few differences between treated and control rats in the food intake, and the overall consumption was only reduced by 1–2%. A similar effect was evident in the values for water intake (Table 3), there being no consistent effect at the 600 ppm dietary level, an overall reduction of 8 and 2% in males and females respectively at the intermediate level and 20% reductions at the top level. The quantities of CHAH consumed are shown in Table 2, the average intake during the experiment being 24, 82 and 300 mg/kg/day by the males for the three dietary levels and 35, 120 and 440 mg/kg/day by the females.

During the first year of the study, the haemoglobin concentrations of the females given 6000 ppm CHAH were consistently reduced by 6–8% compared with the controls and at wk 31 and 52 this was accompanied by reduced values for the packed cell volume (Table 4). There were no similar significant effects later in the study or in the males. Indeed at wk 104 the haemoglobin levels of the treated males, at all levels, were increased compared with the controls. Changes in the leucocytes were confined to lower total counts at wk 52 in male rats given 6000 ppm CHAH and in all treated males at wk 104 and to changes in the ratio of lymphocytes to neutrophils at the highest level in both sexes at wk 80 and 104. Calculation of the total numbers of the different types of leucocytes in the 6000-ppm males showed that

Table 1. *Cumulative mortality of rats given diets containing 0–6000 ppm cyclohexylamine hydrochloride for 2 yr*

Sex and dietary level (ppm)	No. of rats dead at wk										
	35	50	60	70	80	85	88	92	96	100	104
Male											
0	0	0	2	4	6	9	11	14	18	19	24 (14)
600	0	0	1	3	5	7	10	11	14	16	21 (8)
2000	2	3	5	7	9	10	11	13	14	14	18 (9)
6000	1	1	3	3	3	3	3*	3**	4***	5***	5*** (1)
Female											
0	0	1	2	4	10	10	12	14	14	15	16 (8)
600	1	2	2	2	3*	5	5	5*	7	7	10 (5)
2000	0	0	1	1	2*	2*	2**	2**	3**	4**	4*** (1)
6000	0	0	0	0*	0**	0**	1**	2**	5*	6*	7* (6)

The figures are the total numbers of animals dead or killed *in extremis* from groups of 48, except that at wk 104, the numbers of animals in which no histopathological examination was possible are given in parentheses.
The figures marked with asterisks differ significantly (chi-square test) from those of controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

there was a reduction in both neutrophilic polymorphonuclear cells and lymphocytes although the numbers involved were greater (1.9×10^3) for the former cell type than for the lymphocytes (1.0×10^3), representing reductions of 58 and 26% of the total circulating cells of the two types in the controls.

Apart from a reduced lactic dehydrogenase activity at the highest dietary level, the analyses of serum from the females after 2 yr showed no differences between treated and control rats (Table 5). In males, however, the serum-urea concentrations were significantly lower than the control value at all dose levels and the albumin concentrations were higher at the two higher levels (2000 and 6000 ppm). No abnormal constituents were found in the urines from any group. The urine produced in a 6-hr period without water by females given 6000 ppm CHAH was of a lower specific gravity than that of the controls (Table 6), although a similar effect after more prolonged dehydration was seen only at wk 13. Less urine was produced by treated rats following a water load.

There were statistically significant differences compared with the controls in the weights of all organs (Table 7) although most of these differences were not

Table 2. *Body weight and compound intake of rats given diets containing 0–6000 ppm cyclohexylamine hydrochloride for 2 yr*

Sex and dietary level (ppm)	Value at wk										
	0†	2	12	20	32	44	56	68	80	92	101
						Body weight (g)					
Male											
0	130	232	470	547	596	648	692	721	735	709	690
600	134	165***	447**	506***	556**	609*	653*	677*	680*	672	644
2000	133	176**	418***	474***	516***	556***	594***	617***	626***	605***	593***
6000	130	156***	343***	380***	396***	421***	449***	466***	473***	464***	474***
Female											
0	112	165	274	295	320	347	370	394	427	425	429
600	112	144***	242***	265***	283***	303***	328***	350***	373***	383*	380**
2000	112	151***	234***	256***	263***	278***	288***	306***	321***	324***	327***
6000	111	140***	209***	214***	219***	231***	234***	236***	237***	239***	252***
						Intake of cyclohexylamine hydrochloride (mg/kg/day)					
Male											
600	—	71	33	24	19	24	21	17	17	17	19
2000	—	201	113	80	73	89	71	65	56	67	77
6000	—	545	466	285	274	317	268	211	212	268	257
Female											
600	—	80	42	36	33	38	34	28	26	30	31
2000	—	229	174	111	121	134	125	113	90	109	124
6000	—	713	574	401	431	459	440	327	299	424	427

† Value before feeding test compound.
The body weights are mean values for all surviving animals and those marked with asterisks differ significantly (Student's *t* test) from those of the controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
The figures for compound intake are calculated from data on body weight and food consumption.

Table 3. *Food and water consumptions of rats given diets containing 0–6000 ppm cyclohexylamine hydrochloride for 2 yr*

Sex and dietary level (ppm)	Consumption at wk										
	0†	2	12	20	32	44	56	68	80	92	101
						Food consumption (g/rat/day)					
Male											
0	17·3	23·4	28·3	19·5	17·5	28·6	22·2	20·5	20·6	18·6	22·3
600	17·2	25·0	24·6	20·0	17·7	24·6***	22·9	19·3*	19·7	19·4	20·3
2000	18·3	21·9*	23·5	19·0	18·8	24·8***	21·1	20·0	17·5*	20·2	22·9
6000	17·5	19·8***	26·6	18·0*	18·1	22·2***	20·1*	16·4***	16·7*	20·8	20·3*
Female											
0	14·3	17·4	19·1	14·7	14·0	20·6	17·6	17·4	16·5	19·9	20·2
600	13·3	19·1	16·8	15·8*	15·4	19·3	18·6	16·5	16·2	18·8	19·7
2000	15·4	17·3	20·3	14·3	16·0	18·7**	18·0	17·3	14·4*	17·7**	20·3
6000	15·4	16·6	20·0	14·3	15·7	17·7**	17·1	12·9***	11·8***	16·9**	17·9*
						Water consumption (ml/rat/day)					
Male											
0	21·7	29·4	30·5	29·4	30·5	29·2	31·0	34·8	36·5	48·4	47·7
600	23·5	27·0	32·2	33·9	36·0	31·8	31·6	31·3***	34·9	36·2	39·2*
2000	22·8	25·9***	30·3	29·8	33·5	32·1	30·6	31·0**	27·6*	37·9*	34·8**
6000	22·2	23·6***	29·0	25·6*	30·1	27·5	28·1	24·8***	26·1**	29·6***	27·4***
Female											
0	22·4	25·0	28·4	27·2	28·3	31·5	27·8	28·8	35·1	40·2	33·3
600	20·8	20·8**	26·5	31·1	30·8	32·8	29·3	30·0	31·4	38·2	33·8
2000	22·4	23·3*	26·5	26·2	30·7	31·2	31·7	31·6	31·2***	37·8	35·0
6000	22·1	20·7***	22·1***	19·7***	28·3	24·1*	26·9	24·5	21·1**	27·8*	25·9*

† Value before feeding test compound.
The figures are means from 12 cages of rats and were measured over the 24-hr (food) or 48-hr (water) period preceding the measurement of body weight. Those figures marked with asterisks differ significantly (Student's t test) from those of controls: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

evident when the weights were expressed relative to body weight. Both the weights and relative organ weights of the liver, spleen and kidneys in males fed the diet containing 6000 ppm CHAH were lower than the control values. Lower values for the organ weights but higher values for the relative weights were found in the case of the stomach in males at 6000 ppm, small intestine in females at 2000 and 6000 ppm, caecum in both sexes at 6000 ppm and females at 2000 ppm, and the thyroid in females at 2000 and 6000 ppm.

Many of the lesions encountered in the histopathological examination (Table 8) were present with a markedly lower frequency in the rats given 2000 or 6000 ppm CHAH, than in the controls. This was particularly noticeable in the incidences of myocardial fibrosis, mild hepatic changes, hyperplasia of the parathyroids and glomerulonephrosis. With the latter lesion, not only was the total number of affected rats reduced but there was a marked increase in the relative frequency of mild changes and a parallel reduction in the number of rats with the more severe lesions. Increases in the incidence of histopathological changes were restricted to the lungs, where significantly more of the rats given 6000 ppm CHAH had alveoli with foamy macrophages, and to the testes. In the latter organ the incidence of bilateral atrophy was statistically greater in the rats given 6000 ppm CHAH than in the controls. At the intermediate level of CHAH (2000 ppm) there was an increase in the incidence of testes with tubules showing few or no spermatids. There were no statistically significant increases in the incidence of testicular lesions in the rats given 600 ppm CHAH.

The overall number of tumours found (Table 9) was lower in the rats given 6000 ppm than in the controls; indeed, most of the tumours occurred in controls alone or with a similar frequency in treated and control rats. Only three tumours were present in rats given 6000 ppm CHAH without parallel findings in controls. These were a basal-cell carcinoma of the skin and an osteosarcoma of the skull in males and a glioma in the brain of a female. A range of tumours was identified in rats given the lower levels of CHAH without comparable findings in the controls or the highest treatment level. These were reticulum-cell neoplasms of the intestine (in two males at 2000 ppm) and uterus (one rat at 2000 ppm), a pancreatic lipoma (in a female at 600 ppm), adrenal cortical-cell tumours (two at 600 and one at 2000 ppm in females), lymphosarcomas (in males at 600 and 2000 ppm), subcutaneous lipomas (in two males at 600 ppm), a papillary cystadenoma of the salivary gland (in a female at 600 ppm), a sarcoma of the rib (in a male at 2000 ppm), an ovarian adenoma (at 600 ppm), uterine adenocarcinomas (two at 600 ppm), a uterine fibrosarcoma (at 2000 ppm), a uterine squamous-cell carcinoma (at 600 ppm), a prostatic adenocarcinoma (at 600 ppm) and benign testicular interstitial-cell tumours (two at 600 ppm and one at 2000 ppm).

DISCUSSION

The generally more healthy appearance and longer survival of the rats given CHAH, especially at the higher levels, can be directly attributed to their voluntary limitation of food intake, associated with a diet

Table 4. *Haematological findings at various intervals in rats given diets containing 0–6000 ppm cyclohexylamine hydrochloride for 2 yr*

Sex and dietary level (ppm)	No. of rats examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	L	M
					Wk 14					
Male										
0	10	15·4	48	8·33	0·8	11·0	12	2	85	1
2000	10	15·6	49	8·15	—	13·0	—	—	—	—
6000	10	14·7	48	7·71	0·7	11·7	14	2	83	1
Female										
0	10	15·3	45	7·71	0·8	8·8	12	1	86	1
2000	10	15·3	46	7·38	—	10·5	—	—	—	—
6000	10	14·1*	46	7·04	0·6	9·4	10	1	88	1
					Wk 31					
Male										
0	10	14·6	47	7·48	0·6	10·2	15	1	83	1
2000	10	14·8	48	7·91	—	10·6	—	—	—	—
6000	10	14·6	46	7·15	0·6	10·9	12	1	86	1
Female										
0	10	15·8	47	7·08	0·8	9·8	10	1	88	1
2000	10	16·1	47	7·67	—	9·3	—	—	—	—
6000	10	14·6**	44**	7·85	0·6	9·7	11	0	88	1
					Wk 52					
Male										
0	10	14·5	51	7·02	0·3	11·9	19	1	80	0
2000	10	14·0	51	7·24	—	12·1	—	—	—	—
6000	10	13·8	49	6·72	0·7	9·7*	16	2	81	1
Female										
0	10	15·4	49	7·39	0·3	7·6	17	1	82	0
2000	10	15·2	47	7·15	—	7·4	—	—	—	—
6000	10	14·5**	45***	7·28	0·8	8·3	24	1	74	1
					Wk 80					
Male										
0	10	15·2	—	—	—	—	42	2	53	3
2000	10	16·0	—	—	—	—	—	—	—	—
6000	10	15·3	—	—	—	—	33**	2	61*	4
Female										
0	10	15·3	—	—	—	—	43	1	53	3
2000	10	14·3	—	—	—	—	—	—	—	—
6000	10	15·0	—	—	—	—	32**	1	65***	2
					Wk 104					
Male										
0	28	13·6	40	6·94	1·5	7·1	42	2	53	3
600	29	14·5	41	6·35	—	5·6*	—	—	—	—
2000	31	15·2*	43	7·24	0·7	5·2**	38	2	58	2
6000	42	15·9***	45**	7·52	0·6*	4·6***	33**	2	61*	4
Female										
0	29	14·4	43	6·79	1·2	3·2	43	1	53	3
600	37	14·6	42	6·74	—	4·5*	—	—	—	—
2000	44	14·2	42	6·82	1·0	2·9	41	2	55	2
6000	41	15·0	43	7·14	1·0	2·6	32**	1	65***	2

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

The figures are means for the values from the number of animals shown and those marked with asterisks differ significantly (Student's t test) from those of the controls: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

that has been shown to be unpalatable (Gaunt *et al.* 1974). This phenomenon has been demonstrated previously, for example Ross & Bras (1971) restricted the food intake of rats to approximately 30% of the *ad lib.* value and showed markedly longer survival. Similarly, Simms (1967) reported a 25 and 39% increase in the median life span of males and females, respectively, when the food intake was restricted by 46%. In view of this report of a greater effect in females, it is of interest that the survival was affected more in females than in males in the present experiment, significant differences from controls, in terms of the number of rats dead, being evident in males only at the highest level but in females also at the intermediate level.

The finding of a reduced food intake and an associ-

Table 5. *Results of analysis of the serum of rats given diets containing 0–6000 ppm cyclohexylamine hydrochloride for 2 yr*

Sex and dietary level (ppm)	GOT (IU)	GPT (IU)	LDH (IU)	Glucose (mg/100 ml)	Urea (mg/100 ml)	Total protein (g/100 ml)	Albumin (g/100 ml)
Male							
0	44 (20)	13 (21)	931 (17)	164 (23)	48 (15)	7·03 (23)	2·97 (23)
600	44 (21)	12 (24)	968 (16)	152 (23)	30 (17)*	6·64 (25)	3·16 (25)
2000	43 (27)	13 (30)	853 (24)	149 (28)	32 (25)*	6·62 (27)	3·34 (24)*
6000	41 (26)	13 (34)	872 (26)	162 (25)	23 (25)***	7·08 (26)	4·09 (23)***
Female							
0	46 (24)	15 (26)	801 (14)	183 (24)	23 (21)	6·87 (21)	4·24 (19)
600	43 (25)	12 (33)	811 (16)	172 (19)	21 (17)	6·65 (18)	3·91 (19)
2000	35 (8)	11 (8)	671 (7)	174 (16)	20 (16)	6·63 (16)	4·61 (16)
6000	37 (5)	10 (6)	450 (4)**	174 (8)	21 (8)	6·66 (8)	4·21 (8)

The figures are means for the number of estimations shown in parentheses and those marked with asterisks differ significantly (Student's t test) from those of the controls: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

ated failure to gain weight at the same rate as the controls was expected in the present experiment in view of the findings of Gaunt *et al.* (1974) using the same dietary concentrations over a shorter period. The reductions in water intake during the early stages of the experiment were also in keeping with the results of the earlier work (Gaunt *et al.* 1974); and were probably due to the reduced food intake since the two have been shown to be related (Cizek & Nocenti, 1965; Strominger, 1947). The second phase of lower water intake by the treated rats, from wk 60 onwards, is more likely to be related to differences between the groups in the degree of kidney damage. It was noticeable that the intake in the treated animals, particularly at the highest level, remained constant, while the intake of the controls increased over this period. At the end of the study the degree of glomerulonephrosis was considerably less in the treated animals than in the controls. The severely affected kidneys were likely to have been less efficient in terms of concentrating urine, resulting in a greater water loss from the blood. As it has been suggested that water intake is determined by plasma osmolarity (Hatton & Bennett, 1970), such an increase in water loss would result in an increase in water intake.

The differences in serum urea and albumin concentration are equally likely to be dependent on the differences in the condition of the kidney. Increased

Table 6. *Results of renal concentration and dilution tests and of urinary cell excretion in rats given diets containing 0 or 6000 ppm cyclohexylamine hydrochloride for up to 1 yr*

Sex and dietary level (ppm)	Concentration test: Specific gravity 0–6 hr	Concentration test: Specific gravity 16–20 hr	Concentration test: Volume (ml) 0–6 hr	Concentration test: Volume (ml) 16–20 hr	Dilution test (2 hr): Specific gravity	Dilution test (2 hr): Volume (ml)	Cell excretion (10^3/hr)
Wk 13							
Male							
0	1·032	1·050	3·1	2·3	1·003	11·5	13·4
6000	1·032	1·049	4·3	2·2	1·002	9·8	8·9
Female							
0	1·051	1·063	1·1	1·1	1·004	5·6	6·3
6000	1·048*	1·044*	1·5	1·6	1·002	5·8	5·3
Wk 26							
Male							
0	1·057	1.072	2·3	0·7	1·002	7·8	4·9
6000	1·048	1·064	1·4	0·6	1·003	4·4*	3·2
Female							
0	1·049	1·076	1·6	0·3	1·002	8·4	5·0
6000	1·029**	1·077	1·8	0·2	1·004**	3·7**	3·2
Wk 52							
Male							
0	1·060	—	4·7	—	1·006	13·3	5·8
6000	1·046	—	2·1	—	1·005	9·3*	3·8
Female							
0	1·054	1·078	1·4	0·4	1·004	8·8	6·8
6000	1·036*	1·063	0·9	0·4	1·005	3·6*	2·4**

The figures are means for groups of ten rats and those marked with asterisks differ significantly (Ranking method of White, 1952) from those of controls: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

Table 7. *Organ weights and relative organ weights of rats given diets containing 0–6000 ppm cyclohexylamine hydrochloride for 2 yr*

Sex and dietary level (ppm)	No. of rats examined	Organ: Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads††	Pituitary†	Thyroid†	Terminal body weight (g)
Organ weight (g)														
Male														
0	21	2·13	1·78	16·74	1·89	4·52	2·84	10·75	1·94	97	3·65	14·8	41	646
600	24	2·19	1·67	15·65	1·67	3·97*	2·90	10·15	1·51	94	3·56	13·3	40	623
2000	28	2·19	1·50***	13·71***	1·50*	3·50***	2·80	9·03***	1·53	87	3·33***	14·9	38	575**
6000	38	2·13	1·22***	10·20***	0·97***	2·48***	2·56**	7·67***	1·36*	73**	2·51***	11·8*	30***	451***
Female														
0	32	1·93	2·16	11·23	1·78	2·64	1·60	8·06	1·24	89	126	16·5	32	399
600	38	1·95	1·19**	9·90*	1·05	2·41	2·11	7·62	1·15	77	128	17·1	29	360*
2000	44	1·98	1·03***	8·60***	0·85***	2·06***	2·06	7·23**	1·11*	76**	136	15·0	30	311***
6000	41	1·95	0·88***	6·88***	0·67***	1·68***	2·05	6·83***	0·98***	61***	126	11·8***	22***	240***
Relative organ weight (g/100 g body weight)														
Male														
0	21	0·34	0·28	2·65	0·29	0·72	0·45	1·69	0·24	14·9	0·56	2·3	6·7	
600	24	0·36	0·27	2·64	0·28	0·66	0·48	1·65	0·25	15·7	0·57	2·1	6·4	
2000	28	0·39**	0·27	2·43	0·27	0·63	0·50	1·60	0·27	15·5	0·59	2·6	6·7	
6000	38	0·48**	0·27	2·32*	0·21***	0·56***	0·57***	1·71	0·30***	16·6	0·56	2·6	6·8	
Female														
0	32	0·50	0·35	2·86	0·30	0·66	0·55	2·05	0·32	23·4	32·6	4·3	7·9	
600	38	0·56*	0·34	2·81	0·30	0·70	0·61	2·20	0·33	22·2	36·1	4·7	8·1	
2000	44	0·65***	0·34	2·79	0·28	0·67	0·68***	2·37**	0·36*	24·8	43·8**	4·6	9·8***	
6000	41	0·82***	0·37	2·87	0·28	0·70	0·86***	2·86***	0·42***	25·7	54·4***	5·0	9·1***	

† Values for organ weights and relative organ weights expressed in mg and mg/100 g body weight respectively.
†† Values of female gonads expressed in mg and mg/100 g body weight respectively.
The figures are means for the numbers of rats shown and those marked with asterisks differ significantly (Student's *t* test) from those of controls: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

Table 8. *Histopathological lesions in rats given diets containing 0–6000 ppm cyclohexylamine hydrochloride for 2 yr*

	Incidence of lesion							
	Males given dietary level (ppm)				Females given dietary level (ppm)			
	0	600	2000	6000	0	600	2000	6000
Tissue and finding — No. examined....	34	40	39	46	38	43	47	41
Heart								
Myocardial fibrosis	22	24	20	24	20	19	8***	9**
Medial hypertrophy of coronary arteries	4	1	0*	0*	4	1	0*	0*
Endocarditis	0	0	0	0	0	0	0	1
Calcification	2	2	0	0	0	0	0	0
Lungs								
Alveoli with foamy macrophages	6	8	12	19*	5	13	8	21***
Alveoli with thickened walls	15	13	14	19	14	13	15	14
Emphysema	13	12	11	12	9	8	19	16
Leucocytic infiltration	3	2	4	3	5	10	1	6
Granuloma	0	1	1	2	5	5	1	2
Calcification	4	3	0*	1	0	0	0	1
Liver								
Bile-duct hyperplasia	15	15	17	17	26	27	28	22
Bile-duct dilation	1	0	0	0	1	0	3	2
Mild fatty change or vacuolated cells	17	21	15	13*	16	16	2***	3**
Nodular hyperplasia	2	1	0	2	0	0	0	1
Leucocytic infiltration	1	6	3	1	7	5	2	2
Extramedullary haemopoiesis	0	1	1	0	1	0	0	1
Haemangioma	3	1	1	0	0	0	3	0
Kidney								
Mild glomerulonephrosis	4	10	10	16*	13	20	16	17
Moderate glomerulonephrosis	8	9	13	12	19	13	10	0***
Severe glomerulonephrosis	20	18	13*	0***	4	5	1	0*
Total with glomerulonephrosis	32	37	36	28***	36	38	27***	17***
Mineral deposits	2	1	0	1	2	2	3	5
Stomach								
Dilated glands	7	10	13	12	10	12	22	10
Calcification	2	3	0	0	0	0	0	0
Small mucosal haemorrhages	0	1	1	1	0	0	0	0
Small intestine								
Leucocytic infiltration	0	0	0	0	1	2	1	0
Pancreas								
Giant islets	2	4	1	2	1	0	0	0
Medial hypertrophy of arteries	3	3	2	1	4	2	0*	1
Thyroid								
Brachial rest	0	0	1	1	0	1	1	0
Numerous small follicles	3	1	3	1	0	0	0	0
Large follicles	2	2	2	0	3	2	9	1
Colloid cysts	1	3	1	0	1	0	0	0
Hyperplasia of parathyroids	7	3	1	0**	7	4	3	5
Adrenals								
Vacuolation of cortical cells	11	13	12	12	3	7	12*	8
Downgrowth of spindle cells	2	0	3	3	1	0	0	2
Haemorrhage	0	0	0	0	7	5	11	2*
Cysts	0	0	0	0	10	11	16	2**
Pituitary								
Cysts	1	1	1	1	0	2	0	1
Hyperplasia	2	4	4	4	6	5	5	2
Haemorrhage	0	2	0	0	1	2	6	5
Spleen	6	8	9	7	10	8	10	1**
Extramedullary haemopoiesis	4	6	3	9	14	15	25	25
Haemosiderin	7	5	10	5	1	4	0	3
Congestion								
Lymph nodes	4	4	7	11	0	2	6*	2
Haemorrhage	2	1	0	0	0	0	0	0
Hyperplasia								

Table 8 (continued)

Tissue and finding		Incidence of lesion							
		Males given dietary level (ppm)				Females given dietary level (ppm)			
		0	600	2000	6000	0	600	2000	6000
	No. examined....	34	40	39	46	38	43	47	41
Urinary bladder									
Areas of thickened epithelium		1	6	4	1	5	4	3	2
Protein adherent to epithelium		3	3	4	0*	0	0	0	0
Submucosal lymphocyte infiltration		0	0	1	1	3	1	1	0
Skin									
Hyperplasia and hyperkeratosis		2	1	1	1	3	1	0	1
Nodules of granulation tissue (on plantar surface of feet)		3	2	2	1	2	0	0	0
Ovary									
Cystic		—	—	—	—	11	13	11	14
Uterus									
Increase in stroma		—	—	—	—	5	3	2	1
Cystic endometrial hypertrophy		—	—	—	—	5	5	12	13
Fibroid polyp		—	—	—	—	2	2	5	2
Mammary gland									
Galactocoel		—	—	—	—	2	1	2	0
Testes									
Amorphous eosinophilic interstitial material		12	17	14	10	—	—	—	—
Increase of interstitial cells		1	0	5	1	—	—	—	—
Periarteritis		7	4	4	1*	—	—	—	—
Oedema		0	0	3	0	—	—	—	—
Atrophy—unilateral		2	1	4	4	—	—	—	—
Atrophy—bilateral		0	2	2	18***	—	—	—	—
Numerous atrophic tubules		3	7	4	4	—	—	—	—
Tubules with reduced activity		12	8	10	1***	—	—	—	—
Tubules with few or no spermatids		2	6	10*	7	—	—	—	—
Calcium deposits in tubules		1	1	5	10*	—	—	—	—

The figures represent the incidence of the finding in the number of animals shown. Those marked with asterisks differ significantly (chi-square test) from those of the appropriate controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

serum-urea concentration is accepted as an index of severe renal malfunction (Sharratt, 1970), yet in the present experiment there was a dose-related decrease in the urea concentrations. In view of the fact that the degree of degenerative renal change was less in the treated animals, these differences of urea level may be taken to reflect a more efficient renal function. Regarding the higher serum albumin concentrations in the treated male rats, it is known that an increasing proteinuria in ageing rats, especially males, coincides with the development of glomerulonephrosis and that the proportion of albumin in this excreted protein increases (Perry, 1965). This author also showed a parallel decrease in the proportion of albumin in the serum protein. Since there was a lower incidence of nephrosis in the rats given the higher levels of CHAH in the present study and in view of the work of Simms (1967) showing that the onset of renal damage was markedly delayed in rats fed a limited quantity of food, it is likely that the increased albumin concentrations are a result of decreased kidney damage due to reduced growth. As the degree of renal change in the treated rats was less than in the controls, the finding of impaired renal concentrating ability in females given the highest dose of CHAH must, at present, be related to the effects of treatment, particularly since a similar effect was reported by Gaunt *et al.* (1974). However, even this conclusion must be accepted with caution since Sharratt (1970) cited evidence that restricted dietary intake in rats could lead to such changes in the results of concentration tests.

Most of the differences between the organ weights of control and CHAH-treated rats were a reflection of the differences of body weight, since they were no longer apparent when the weights were expressed relative to body weight. The increased relative weights of the brain were also attributable to the changes in body weight, since it has been shown that following limitation of food intake to 87·5% of the *ad lib.* value (a figure comparable with the reductions in the present study) the brain weights were normal but the relative values were increased (Schwartz, Tornaben &

Table 9. *Incidence of tumours in rats given diets containing 0–6000 ppm cyclohexylamine hydrochloride for 2 yr*

Tissue and tumour	Males 0	Males 600	Males 2000	Males 6000	Females 0	Females 600	Females 2000	Females 6000
	Incidence of tumour							
	Males given dietary level (ppm)				Females given dietary level (ppm)			
	0	600	2000	6000	0	600	2000	6000
No. examined....	34	40	39	46	38	43	47	41
Lung								
Adenoma	1	1	0	0	0	0	0	0
Kidney								
Adenoma	1	0	1	0	0	0	0	0
Lipoma	0	1	0	0	1	0	0	0
Liver								
Reticulum cell neoplasm	0	0	0	0	1	1	0	0
Intestine								
Adenoma	2	0	0	0	0	0	0	0
Fibroma	0	0	0	0	1	0	0	0
Reticulum cell neoplasm	0	0	2	0	0	0	0	0
Pancreas								
Insuloma	2	0	2	0	0	0	0	0
Exocrine adenoma	2	2	0	0	0	0	0	0
Lipoma	0	0	0	0	0	1	0	0
Adenocarcinoma	1	0	0	0	0	0	0	0
Thyroid								
Adenoma	6	4	1	5	0	3	2	0
Adrenal								
Medullary cell tumour	5	0	2	1	0	0	1	0
Cortical cell tumour	0	0	0	0	0	2	1	0
Ganglioma	0	0	0	0	1	0	0	0
Pituitary								
Chromophobe adenoma	4	5	7	1	10	7	11	6
Lymph nodes								
Reticulum cell neoplasm	1	1	0	0	0	1	0	0
Generalized lymphosarcoma	0	1	1	0	0	0	0	0
Thymus								
Lymphosarcoma	0	0	0	0	1	0	0	0
Thymoma	0	0	0	0	1	1	1	0
Lymphoma	0	0	0	0	1	1	0	0
Subcutaneous tissue								
Fibroma	1	0	0	0	0	0	0	1
Lipoma	0	2	0	0	0	0	0	0
Fibrosarcoma	1	1	1	0	2	0	0	0
Salivary gland								
Papillary cystadenoma	0	0	0	0	0	1	0	0
Skin								
Basal cell carcinoma	0	0	0	1	0	0	0	0
Squamous cell carcinoma	0	0	0	0	1	0	0	0
Skeleton								
Osteosarcoma (skull)	0	0	0	1	0	0	0	0
Osteo- or chondrosarcoma (rib)	0	0	1	0	0	0	0	0
Peritoneum								
Lipoma	0	0	0	0	3	1	0	0
Brain								
Glioma	0	0	0	0	0	0	0	1
Mammary gland								
Fibroadenoma	0	0	0	0	5	9	7	2
Adenocarcinoma	0	0	0	0	2	0	1	2
Ovary								
Granulosa cell tumour	—	—	—	—	1	0	2	1
Adenoma	—	—	—	—	0	1	0	0
Uterus								
Fibroadenoma	—	—	—	—	2	0	0	0
Adenocarcinoma	—	—	—	—	0	2	0	0
Fibrosarcoma	—	—	—	—	0	0	1	0
Squamous cell carcinoma	—	—	—	—	0	1	0	0
Reticulum cell neoplasm	—	—	—	—	0	0	1	0
Prostate								
Adenocarcinoma	0	1	0	0	—	—	—	—
Testes								
Benign interstitial cell tumour	0	2	1	0	—	—	—	—
Malignant interstitial cell tumour	1	1	0	0	—	—	—	—

The figures represent the incidence of the tumour among the number of rats shown.

Boxill, 1973). Increases in the relative weight of stomach, small intestine, caecum and ovary comparable with those in the present study were found in a short-term study by Gaunt *et al.* (1974). In that experiment, as in the present work, there was no histopathological evidence to suggest any damage to these organs so that these changes are likely to be a reflection of the body-weight differences rather than of a treatment-mediated change. The lower relative kidney weights in the CHAH-treated animals can be related to the lack of pathological change in these rats since a marked increase in size of the affected organs is said to be characteristic of the rat nephrosis (Berg, 1967). The lower heart weights are also likely to be a result of the lack of pathological change in these rats since the degree of myocardial fibrosis was significantly less in the hearts of animals given CHAH than in the controls. This fibrosis in ageing rats has been shown to be accompanied by a hypertrophy probably secondary to the hypertension associated with the developing renal changes (Berg & Harmison, 1955).

The reduced weight and relative weight of the spleen can also be related to the effects of a reduced food intake. There was a marked leucopenia in the male animals, confirming the previous observations (Gaunt *et al.* 1974) of this effect. A leucopenia has been related to the intake of severely reduced quantities of food in rats (Shukers & Day, 1943; Zbinden, 1963). Such a reduction in white cells will involve a reduction in the number of lymphocytes as these cells constitute the majority of the leucocytes in rats. Approximately 50% of the cells of the rat spleen have been shown to be lymphocytes (Jandl, Files, Barnett & MacDonald, 1965) and it is possible that a prolonged lower production of these cells could result in the observed decreases in splenic weight.

The increased relative thyroid weights in females given 2000 or 6000 ppm CHAH may have resulted from the treatment, since Gaunt *et al.* (1974) found increased oxygen consumptions in rats given 6000 ppm CHAH, but this association between treatment and elevated thyroid weight is tenuous as there was no comparable effect in the males.

In the short-term study on CHAH (Gaunt *et al.* 1974), lowered testis and relative testis weights were an outstanding finding. In the present study, although the fresh weight of the testes was lower than that in controls in males given the two higher dose levels, differences were not apparent when the values were expressed relative to body weight. However, the histopathological examination showed that, at the highest dietary level, the testes were sharply divided into those with no observable damage or only slight changes and those with marked effects. Calculation of the mean weights for rats with these various degrees of histopathological effect showed that the fresh and relative weights were 1·49 g and 0·36 g/100 g body weight respectively for the affected rats compared with 3·19 g and 0·70 g/100 g body weight for the unaffected rats. These values differed significantly from each other ($P < 0{\cdot}001$). Compared with the controls, both values for the affected animals but only the absolute value for the histologically unaffected rats differed significantly. Hence the results of this study have confirmed the earlier work (Gaunt *et al.* 1974) in that a proportion of the rats given CHAH showed testicular changes associated with a reduced absolute and relative testis weight. This is in sharp contrast with the findings in mice given comparable intakes of CHAH for 80 wk, in which animals there were no testicular changes (Hardy *et al.* 1976). This may suggest that the testicular changes are species specific although, at present, there are insufficient data available from various species to be clear on this point. In addition no evidence is available to suggest why some animals show a marked effect whilst others are undamaged.

The observation of reduced haemoglobin concentrations in the females given 6000 ppm during the first 18 months of the study confirms this finding at 13 wk in a short-term study (Gaunt *et al.* 1974) although in this earlier work the effect was seen in both sexes. This finding must be attributed to treatment since the available evidence (Schwartz *et al.* 1973) indicates that dietary restriction leads to increased rather than decreased haemoglobin concentrations. The increases in haemoglobin concentration seen at 2 yr in males given CHAH may well be attributable to this effect of reduced food intake and the subsequent improvement in the general condition of the rats.

The majority of the changes in the incidence of histopathological findings can also be related to the reduced food intake and the failure to grow to the point of obesity. Indeed it may be that the failure to develop severe glomerulonephrosis is the primary effect with secondary sparing effects in other organs. On the basis of the work of Simms (1967) and of Saxton & Kimball (1941) it is established that animals not becoming obese do not readily develop severe renal changes. The latter workers adjusted the diet to maintain the weight of rats at 90% of control values and reduced the incidence of nephrosis from 25 to 3% in animals killed randomly during the study for histopathological examination and from 52 to 37% in those that died. They also presented evidence that the primary effect is the deposition of protein in the renal tubules, and since the excretion of protein is decreased by undernutrition (Schwartz *et al.* 1973) this could be a primary effect. On the basis of these considerations, the finding of a much-reduced renal damage in the present study was not surprising. A decrease in myocardial changes and in periarteritis has been reported by Simms (1967) in rats maintained on reduced quantities of food. In addition, the normal senescent cardiac changes may be associated with hypertension in the face of advancing renal damage (Berg & Harmison, 1955) so that the lesser degree of cardiac damage in the present experiment was to be expected. Similarly parathyroid hyperplasia has been related to the severity of renal damage (Snell, 1967) and subsequent calcium–phosphorus imbalance so that the lower incidence of parathyroid changes in this experiment can be related to the reduced severity of the kidney damage.

Against this background of a general tendency for a reduced severity in the pathological findings, the presence of an increased incidence of minor pulmonary change, in the form of alveoli with foamy macrophages, in the rats given 6000 ppm CHAH must be attributed to the treatment. The other organ showing an effect was the testis, as was demonstrated in the short-term study in these laboratories (Gaunt *et al.*

1974). There was a marked effect at the highest level of treatment although only approximately half of the rats were affected. At the intermediate level there was a statistically significant increase in the number of rats with less severe changes in the form of tubules with fewer or no spermatids. This effect is less clear-cut, since the presence of similar lesions in control rats suggests that it may occur as a normal senescent change. Nevertheless the magnitude of this increase, combined with the previous evidence of testicular effects at the same level (Gaunt *et al.* 1974), shows that this must be attributed to CHAH. At the lowest level (600 ppm) there were no definite statistically significant increases in the incidence of histopathological changes in the testis. The ageing rat is not a good model for the study of this type of effect, since atrophic changes of various degrees are known to occur in many such animals (Brantom, Gaunt, Hardy, Grasso & Gangolli, 1973; Gellatley, cited from Russfield, 1967). Against this background it is possible that the number of affected animals in the rats given 600 ppm CHAH could be within the expected range. In view of the absence of effect in the testes of young rats at this level (Gaunt *et al.* 1974) and the fact that, in general, the severity of the effect at the higher levels was no greater following the prolonged exposure in the present experiment than after the previous shorter exposure, it seems unlikely that CHAH was exerting an adverse effect on the testes at this low level of treatment.

There is, so far, no evidence to suggest the mechanism by which CHAH exerts its effect on the testis to bring about a reduced weight and atrophic changes. Gellately (cited from Russfield, 1967) pointed out an association between atrophic changes and arterial changes, but this seems unlikely in the present experiment since the incidence of cardiovascular changes was lowest in the treated rats. It might be postulated that testicular changes could be the result of prolonged undernutrition and failure to gain weight. Indeed in animals maintained on a protein-free diet, extensive testicular damage resulted (Boyd, 1970) together with massive reductions in testis weight and relative testis weight. However, with rats maintained on a diet containing 9% protein, compared with a 26% protein control, the testis weight was reduced but the relative weight was increased. The histopathological condition of the testes of the rats given 9% protein was not reported. This suggests that testicular damage and weight changes occur only as a response to extreme dietary deprivation, a fact supported by Schwartz *et al.* (1973) who found no histopathological changes in the testes of rats given half their *ad lib.* intake of normal food for 210 days. These authors found that the relative testis weight was increased. This combination of increased relative weight with no histopathological damage has been encountered in short-term studies in our laboratories under conditions of limited food intake due to unpalatable diets (Colley, Gaunt, Lansdown, Grasso & Gangolli, 1969; Gaunt, Farmer, Grasso & Gangolli, 1967; Gaunt, Sharratt, Colley, Lansdown & Grasso, 1970). Against this background, the finding of testicular damage and lowered relative testis weights is unusual and unlikely to be the outcome of slight or moderate reductions in food intake.

The generally lower incidence of tumours is expected in animals that have not become obese (Ross & Bras, 1971). Most of the tumours were found in the controls alone or in controls and treated rats with similar frequencies; indeed in no case was there a significant increase in tumour incidence in treated rats compared with the controls. This indicates a lack of carcinogenic effect on the part of CHAH and this is supported by the fact that those tumours present only in treated rats can be considered as examples of the spontaneous tumour incidence. Of the three tumour types found solely in the high-level rats, one, a glioma, has been encountered in untreated rats in our laboratories (Brantom *et al.* 1973; Gaunt, Butterworth, Hardy & Gangolli, 1975). The basal-cell carcinoma and osteosarcoma are less common, but have both been reported in untreated rats by Curtis, Bullock & Dunning (1931), and osteosarcoma was found by Ross & Bras (1973).

Those tumours found in the present study in the rats given 600 or 2000 ppm CHAH without comparable observations in the control rats or those given the highest level of CHAH have been either encountered in our own laboratories in untreated rats or in animals given materials judged to be non-carcinogenic or described by other workers. Some are commonly found, for example adrenal cortical tumours and testicular interstitial-cell tumours (Brantom *et al.* 1973; Gaunt *et al.* 1975; Prejean, Peckham, Casey, Griswold, Weisburger & Weisburger, 1973). These authors have also described reticulum-cell neoplasms, lipomas of various sites, lymphosarcoma and salivary-gland adenoma. The remaining three tumours encountered in this study, although less common, have been described by other workers. Ovarian adenomas were described by Snell (1965) and uterine adenocarcinoma by Snell (1965) and Franks (1967), while uterine fibrosarcoma is considered to be spontaneous by Franks (1967) and Prejean *et al.* (1973). In view of this established spontaneous incidence of the tumours, the low incidence of the individual tumours and their absence from the rats given the highest level of CHAH, it is considered that the occurrence of these tumours represents the normal incidence in rats rather than any effect of treatment.

The results of this study have shown that most of the differences between treated and control rats could be attributed to an attenuation of the normal geriatric changes in rats resulting from a failure of the CHAH-treated rats to become obese. Mild anaemia during the first year of the study in those rats given 6000 ppm CHAH was attributed to treatment, as was an increased incidence of minor histopathological changes of the lung and possibly a reduced renal concentrating ability. There were testicular changes attributable to treatment in rats given 2000 or 6000 ppm CHAH but no definite effect at the lowest level. From the data obtained in this study, it is concluded that no carcinogenic effect could be detected in rats given CHAH in their diet at levels up to 6000 ppm. This is similar to the findings in mice given comparable intakes (Hardy *et al.* 1976). The no-untoward-effect level is 600 ppm, on the basis of the effects on the testis. The dose levels used in this study were based on the maximum likely intake of cyclohexylamine from the conversion of cyclamate; hence, if man is

no more sensitive than the rat to these effects, the consumption of cyclamates is unlikely to represent a hazard. However, more work is required to elucidate the mechanisms of the testicular changes before any firm conclusion can be drawn.

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LONG-TERM TOXICITY OF CYCLOHEXYLAMINE HYDROCHLORIDE IN MICE

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Abstract—Groups of 48 male and 50 female mice were fed on diets containing 0 (control), 300, 1000 or 3000 ppm cyclohexylamine hydrochloride for 80 wk. There were no effects attributable to treatment in the number of deaths, rate of body-weight gain, food intake, water intake, haematological examinations or the incidence of tumours. The only histopathological change that could be related to treatment was an increased incidence of minor hepatic changes in the females given 3000 ppm. It is concluded that CHA exerted no carcinogenic effect at dietary levels up to 3000 ppm, while the no-untoward-effect level was 1000 ppm.

INTRODUCTION

Cyclohexylamine is a strongly basic material, which reacts with all acids, including long-chain fatty acids, to form salts. Cyclohexylamine and its salts are used as insecticides, plasticizers, corrosion inhibitors, dry-cleaning soaps, solvents, components of rubber-vulcanizing processes, antistatic agents in cellulose derivatives and intermediates in the production of chemicals such as dyes. However, the discovery (Kojima & Ichibagase, 1966) that the free amine was an *in vivo* metabolite of the cyclamate sweeteners (sodium or calcium salts of cyclohexylsulphamic acid) stimulated a renewed interest in the toxicity of cyclohexylamine.

The data concerning the metabolism of cyclamate to cyclohexylamine as well as those concerning the metabolism and toxicity of cyclohexylamine itself, were reviewed by Gaunt, Sharratt, Grasso, Lansdown & Gangolli (1974). In addition, these workers fed dietary levels of 600, 2000 or 6000 ppm cyclohexylamine hydrochloride to groups of 15 male and 15 female rats for 13 wk and found a reduced rate of body-weight gain together with a reduced food intake in rats given the two higher concentrations. A paired-feeding experiment showed that the reduced food intake did not account fully for the failure to gain weight although no impairment of nutrient absorption was detected. However, measurements of oxygen consumption suggested an increase in the basal metabolic rate. These authors also found that female rats given 6000 ppm cyclohexylamine hydrochloride showed a slight impairment of renal concentrating ability.

An outstanding finding in the study of Gaunt *et al.* (1974) was a reduction in testis weights in the animals given the two higher levels (2000 and 6000 ppm). This was accompanied by histopathological evidence of reduced spermatogenesis, amounting to complete arrest and loss of the germinal epithelium in 40% of the rats given 6000 ppm cyclohexylamine hydrochloride. Despite this effect on the testis, a limited reproduction study showed no statistically significant differences between the offspring of untreated males and of those given 6000 ppm cyclohexylamine hydrochloride, in terms of the number of fertile males or litter size and growth.

The present study was initiated to assess the effects of cyclohexylamine given over prolonged periods to mice. A parallel long-term study in rats is reported separately (Gaunt, Hardy, Grasso, Gangolli & Butterworth, 1976).

Consideration was given to potential human exposure to cyclohexylamine when establishing the doses for this study. Since 50 mg cyclamate/kg/day has been suggested as the maximum acceptable intake in man (Joint FAO/WHO Expert Committee on Food Additives, 1968) and quantitative conversion of this amount would provide a dose of approximately 30 mg cyclohexylamine/kg/day, the lowest level used in the present study was that calculated to provide approximately this daily intake.

EXPERIMENTAL

Materials. The cyclohexylamine hydrochloride (CHAH) used in these studies was prepared by Laporte Industries, Ilford, Essex, from cyclohexylamine conforming to the specification of the British Standards Institution (1968) and was from the same batch as was used for the previous studies in rats (Gaunt *et al.* 1974).

Animals and diet. Weanling mice of the ASH-CS1 strain obtained from a specified-pathogen-free colony were housed in an animal room at 20 ± 1°C with a relative humidity of 50–60%. The basic diet was reground Oxoid breeders diet supplemented with 60 ppm sodium menadione bisulphite. Food and water were freely available.

Experimental design and conduct. Groups of 50 female mice, housed five to a cage, and 48 male mice,

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housed individually, were fed diets containing 0 (control), 300, 1000 or 3000 ppm CHAH for 80 wk. The animals were observed at frequent intervals to monitor their general health and behaviour. Any mouse that appeared to be ill was isolated, to be returned to its cage if its condition improved or killed if there was no improvement. Mice killed or found dead during the study were subjected to a post-mortem examination and samples were preserved for histopathological examination, unless this was precluded by advanced autolysis or cannibalism.

Body weights were measured at intervals throughout the study. At the same intervals the food and water intakes were measured over the 4-day period preceding the day of weighing. The consumptions were measured for six individually caged male mice and six cages each of five females from each group, the same animals being used throughout the experiment.

Blood was collected during wk 13, 26 and 52 from a tail vein of ten mice of each sex from the controls and from the groups given 1000 and 3000 ppm CHAH. At the end of the experiment, blood was collected from a tail vein of all surviving mice immediately before killing. At all four examinations the concentration of haemoglobin was measured in each sample and a blood smear was stained for examination of red cell morphology and enumeration of the various types of leucocytes. This examination was confined to the control mice and those given 3000 ppm CHAH. In addition, the packed cell volume was measured and counts were made of the total erythrocytes and total leucocytes in the samples taken at wk 13, 26 and 52. Preparations for counting reticulocytes were made from the samples taken at wk 13, 26 and 52 but the counts were confined to the control mice and those given 3000 ppm CHAH.

During wk 80–84 all surviving mice were killed by exsanguination under barbiturate anaesthesia following a 24-hr period without food. During a post-mortem study on each mouse, an examination was made for any macroscopic abnormalities and the brain, heart, liver, spleen, kidneys, stomach and small intestine were weighed. Samples of these tissues and of pituitary, salivary gland, thyroid, thymus, adrenal glands, various lymph nodes, pancreas, lung, gonads, colon, caecum, rectum, oesphagus, trachea, skeletal muscle and uterus or seminal vesicle, together with any other tissue appearing to be abnormal were preserved in buffered formalin. The urinary bladders were inflated with 0·5–1·0 ml Bouin's fixative and immersed in the same fixative for 24 hr, after which time the bladders were bisected by a median sagittal incision and examined under a low power (× 10) microscope for gross abnormalities before being transferred to 10% buffered formalin.

All tissues were processed in the usual way for wax embedding and sections were stained with haematoxylin and eosin for microscopic examination.

RESULTS

Some mice from each group died during the study (Table 1), but the numbers dead at any one time were similar in treated and control groups and there was no dose-relationship in the mortality rate. The overall

Table 1. *Cumulative mortality of mice fed diets containing 0–3000 ppm CHAH for up to 80 wk*

	No. of deaths among males at dietary level (ppm) of				No. of deaths among females at dietary level (ppm) of			
Wk no.	0	300	1000	3000	0	300	1000	3000
20	0	0	0	0	0	0	1	0
40	1	0	1	2	2	1	2	1
60	2	3	2	3	5	5	6	3
80	8 (2)	7 (3)	7 (5)	10 (2)	8 (5)	11 (3)	15 (4)	9 (4)

The figures represent the total numbers of mice found dead or killed *in extremis* from groups of 48 males or 50 females. The figures in parenthesis are the numbers on which histopathology was not possible because of autolysis.

Table 2. *Initial body weights and weight gain of mice given diets containing 0–3000 ppm CHAH for up to 80 wk*

Dietary level (ppm)	Initial body weight (g)	Body-weight gain (g) at wk								
		1	4	8	12	16	20	26	52	80
					Males					
0	25·3	3·0	6·6	7·6	9·2	10·4	14·8	18·7	19·9	22·9
300	27·6***	2·0***	5·8	6·7	7·9*	9·5	13·3	16·2*	17·5	20·8
1000	27·6***	1·4***	4·7***	6·2*	7·2**	8·9*	12·3**	15·6*	17·3	18·0**
3000	28·2***	1·2***	4·6***	5·9**	6·7	7·3***	10·6***	14·1***	15·4***	16·6***
					Females					
0	21·0	0·8	3·4	7·1	8·2	9·2	11·5	13·5	14·9	16·2
300	20·6	0·8	4·3	7·1	8·6	9·5	12·2	14·6	15·4	17·5
1000	20·3	0·9	3·7	6·8	8·0	8·7	12·1	13·6	14·7	16·9
3000	20·2	0.9	3·5	7·2	7·5	9·4	12·6	14·0	14·2	15·2

Figures are means for all surviving mice and those marked with asterisks differ significantly (Student's t test) from those of the appropriate controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Table 3. *Relative organ weights of mice given diets containing 0–3000 ppm CHAH for 80 wk*

Sex and dietary level (ppm)	No. of mice examined	Relative organ weights (g/100 g body weight) Brain	Heart	Liver	Spleen	Kidney	Stomach	Small intestine	Terminal body weight (g)
Male									
0	40	1·04	0·57	4·46	0·23	1·57	0·88	4·76	45
300	41	1·05	0·54	4·45	0·28	1·63	0·88	4·72	46
1000	41	1·05	0·60	4·74	0·27	1·65	0·89	4·88	44
3000	39	1·10	0·57	4·39	0·24	1·67	0·88	4·74	43
Female									
0	42	1·36	0·49	4·41	0·34	1·24	1·28	4·89	35
300	40	1·28	0·46	4·24	0·33	1·15	1·10*	4·59	37
1000	33	1·38	0·49	4·26	0·37	1·20	1·16	4·77	35
3000	40	1·37	0·48	4·50	0·34	1·22	1·18	4·88	35

Figures are means for the numbers of mice shown and that marked with an asterisk differs significantly (Student's t test) from that of the corresponding control: $*P < 0{\cdot}05$.

mortality was low, being 17 and 22% in males and females, respectively, at wk 80. The corresponding figures at 1 yr were 3 and 8%, so that most of the animals were exposed to the appropriate level of CHA for prolonged periods.

The gain in body weight (Table 2) was similar in all groups of female mice. There was a statistically significant difference between the mean initial body weight of the control male mice and those of the three treatment groups, the treated groups being heavier. Subsequently there was a difference in the rate of body-weight gain throughout the experiment, with the treated groups having a lower rate of weight gain than the controls. The differences from controls were statistically significant at most examinations in the male mice given 1000 or 3000 ppm CHAH, although the differences were greater at the higher level. There were only occasional statistically significant differences between the controls and the mice given 300 ppm CHAH.

No reductions were detected in the food intake of any CHAH-treated group compared with the controls. The water intake of the females given CHAH was slightly less than that of the controls, although the differences were statistically significant only during wk 1 of the study. On the other hand, the treated males showed a tendency to drink more water than the controls, but there was no dose relationship in this observation.

The stomach weights of all three groups of female

Table 4. *Haematological findings in mice given diets containing 0–3000 ppm CHAH for 52 or 80 wk*

Sex and dietary level (ppm)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	L	M
Wk 52										
Male										
0	10	16·2	50	8·05	1·2	7·41	20	0	79	1
1000	10	16·1	50	8·38	—	9·51	—	—	—	—
3000	10	16·6	51	8·47	1·3	8·95	18	0	81	1
Female										
0	10	16·0	48	8·02	1·2	5·80	15	0	84	1
1000	10	16·3	49	8·28	—	7·63	—	—	—	—
3000	10	15·8	46	7·49	1·9	6·79	21	1	75*	3
Wk 80										
Male										
0	39	14·9	—	—	—	—	33	1	62	4
300	40	14·6	—	—	—	—	—	—	—	—
1000	38	14·5	—	—	—	—	—	—	—	—
3000	38	14·3	—	—	—	—	41*	1	57*	1
Female										
0	42	15·8	—	—	—	—	44	0	54	2
300	39	15·8	—	—	—	—	—	—	—	—
1000	33	15·7	—	—	—	—	—	—	—	—
3000	39	15·4	—	—	—	—	42	0	57	1

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

The figures are means for the numbers of mice indicated and those marked with an asterisk differ significantly (Student's t test) from the appropriate control: $*P < 0{\cdot}05$.

mice given diets containing CHAH were significantly lower ($P < 0.05$) than those of the controls but the mean weights at all three dose levels were the same and there was no similar finding in the males. When the stomach weights were expressed relative to body weight (Table 3), the differences were less marked and the only statistically significant differences ($P < 0.05$) occurred at the lowest level (300 ppm). Compared with the controls, there was an isolated increase in haemoglobin concentration in the blood of the female mice given 1000 ppm CHAH for 13 wk and there were occasional slight changes in the mean ratios of lymphocytes and neutrophilic polymorphonuclear leucocytes at the highest level (3000 ppm) (Table 4). These changes were inconsistent, being an increase in the proportion of lymphocytes in the males at wk 13 (87 compared with 79%; $P < 0.01$) but a decrease at the end of the study (Table 4). There was a similar decrease in the proportion of lymphocytes in the females at wk 52 (Table 4).

Table 5. *Incidence of histopathological lesions (excluding tumours) found in mice fed diets containing 0–3000 ppm CHAH for 80 wk*

Tissue and finding	Incidence of finding in males at dietary level (ppm) of				Incidence of finding in females at dietary level (ppm) of			
	0	300	1000	3000	0	300	1000	3000
No. of mice examined...	46	45	31	46	44	46	42	44
Liver								
Nodular hyperplasia	5	3	10	3	1	3	0	1
Minor changes (cell vacuolation or polyploidy)	13	11	4	13	4	10	10	13*
Foamy macrophages	2	3	6*	7	2	5	4	4
Areas of necrosis or infarction	5	3	3	4	3	5	3	6
Leucocytic infiltration or deposits	4	6	3	3	8	12	7	9
Kidneys								
Glomerulonephrosis	7	6	2	2	12	26**	14	9
Leucocytic infiltration or deposits	7	13	8	13	23	24	15	31
Heart								
Foci of calcification	0	1	0	0	1	0	1	0
Pericarditis	0	0	0	1	0	0	0	0
Lungs								
Leucocytic infiltration or deposits	3	0	1	4	2	2	2	9*
Spleen								
Marked extramedullary haemopoiesis	5	3	2	3	1	2	2	1
Lymphoid hyperplasia	2	0	2	0	1	0	2	1
Amyloid deposition	0	0	0	0	0	1	0	1
Bladder								
Submucosal lymphocytes	1	3	5*	0	10	15	8	10
Protein exudate	1	2	1	2	0	0	0	0
Localized loss of epithelium	0	2	1	0	0	0	0	0
Localized hyperplasia of epithelium	0	0	0	0	0	1	1	1
Skin								
Granuloma	1	0	0	0	0	1	0	0
General								
Lymphoid hyperplasia	0	0	1	1	0	1	1	2
Amyloid	0	0	0	0	0	1	1	1
Ovary								
Cystic	—	—	—	—	5	2	3	0*
Haemorrhage	—	—	—	—	2	3	1	1
Uterus								
Cystic endometrial hyperplasia	—	—	—	—	10	15	14	8
Testes								
Atrophic tubules	2	1	4	4	—	—	—	—
Calcified tubules	1	2	0	3	—	—	—	—
One testis atrophic	1	0	1	0	—	—	—	—

The figures represent the numbers of mice affected among the numbers of mice shown and those marked with an asterisk differ significantly (chi-square test, $*P < 0.05$) from that of the appropriate control.

Low-power microscopic examination of the fixed urinary bladders showed that a few mice from each group, including the controls, had plaques on the surface of the mucosa. Histopathological examination (Table 5) revealed foci of leucocytes, mainly lymphocytes, in the submucosa immediately beneath the epithelium of the bladders of approximately 5% of the males and 25% of the females. The incidence of this lesion was statistically greater than the control value in the males given 1000 ppm CHAH but there was no increased incidence at the higher level. There was a localized hyperplasia of the bladder epithelium in one female mouse from each treated group and isolated areas of epithelial loss in three male mice (two given 300 ppm and one given 1000 ppm CHAH). There were no changes suggestive of neoplasia in any urinary bladder, either at autopsy or at the histopathological examination.

The incidences of most of the histopathological findings (Table 5) were similar in treated and control mice. Lesions found with a statistically higher incidence in the treated animals were mild hepatic changes in the females given the highest dietary level, foamy hepatocytes in males at the intermediate level and glomerulonephrosis in females at the lowest level.

Tumours were found in all groups (Table 6) but there were no statistically significant differences between treated and control mice in the incidences of the individual types of tumour. In addition, the total number of tumours, the total malignant tumours

Table 6. *Incidence of tumours in mice given diets containing 0–3000 ppm CHAH for 80 wk*

	Incidence in males at dietary level (ppm) of				Incidence in females at dietary level (ppm) of			
	0	300	1000	3000	0	300	1000	3000
Tissue and tumour — No. of mice examined...	46	45	31	46	44	46	42	44
Lung								
Adenoma	12	10	4	7	6	9	7	5
Adenocarcinoma	0	0	1	0	0	0	0	0
Adrenal								
Medullary tumour	0	1	0	0	1	1	0	0
Skin								
Squamous-cell carcinoma	0	1	0	0	0	0	1	0
Melanoma	0	0	1	0	0	0	0	0
Subcutaneous tissue								
Fibrosarcoma	0	0	1	1	0	1	0	0
Haemangioendothelioma	1	0	0	0	0	0	0	0
Lymphoreticular system								
Generalized lymphosarcoma	1	0	1	1	0	2	3	2
Reticulum-cell neoplasms:								
main focus, intestine	0	1	1	0	0	0	0	0
main focus, liver	0	0	0	0	1	0	2	0
main focus, lymph node	0	0	0	0	1	2	1	0
main focus, uterus or ovary	—	—	—	—	2	1	0	3
Total...	0	1	1	0	4	3	3	3
Myeloid leukaemia	1	0	0	0	0	0	0	0
Heart								
Rhabdomyosarcoma	0	0	0	1	0	0	0	0
Kidney								
Adenoma	0	2	0	0	0	0	0	0
Adenocarcinoma	0	0	0	1	0	0	0	0
Testis								
Seminoma	0	0	1	0	—	—	—	—
Embryonal-cell tumour	0	0	1	0	—	—	—	—
Intestine								
Adenoma	0	0	0	0	1	0	0	0
Ovary								
Granulosa-cell tumour	—	—	—	—	0	1	3	0
General								
Sarcoma involving pancreas, lung, diaphragm	0	0	0	0	0	0	0	1
Total no. of tumours	17	15	11	11	12	17	17	11
No. of tissues involved	3	5	5	5	4	6	4	3
Total no. of malignant tumours	3	2	6	4	4	6	7	6
No. of tissues involved	2	2	5	4	1	2	2	2
No. of mice with tumours	16	14	10	11	11	15	15	10
No. of mice with multiple tumours	1	1	1	0	1	2	2	1

The figures represent the numbers of mice affected among the numbers of mice shown.

and the numbers of tissues involved were similar in treated and control mice. Some tumours were found in mice given the lower doses of CHAH without any parallel findings in the controls or in mice on the highest level of treatment. These were a pulmonary adenocarcinoma in a male given 1000 ppm, squamous-cell carcinomas of the skin in a male given 300 ppm and a female given 1000 ppm, a melanoma in a male given 1000 ppm, a seminoma and an embryonal-cell tumour in males given 300 ppm and ovarian granulosa-cell tumours at 300 and 1000 ppm. Tumours found at the highest level of treatment without corresponding control findings were a subcutaneous fibrosarcoma (also in a male given 1000 ppm and a female given 300 ppm), a rhabdomyosarcoma of the heart, a renal adenocarcinoma and a generalized sarcoma probably originating from the lung.

DISCUSSION

The lower rate of body-weight gain in the treated male mice may be considered to be related to treatment since a reduced rate of weight gain was a prominent feature of the short-term study with CHAH in rats (Gaunt *et al.* 1974). However, in rats there was evidence of a reduced intake of an unpalatable food whereas, in the present study, there was no evidence for a rejection of the food. In addition, the effect on weight gain was apparent in both sexes of rat, whereas the treated female mice in this experiment gained weight at the normal rate. The initial body weights of the treated male mice were significantly greater than those of the controls and it seems likely that the reduced rate of increase of body weight was related to this higher initial weight rather than to treatment with CHAH. However some effect of treatment cannot be totally ruled out at the highest level (3000 ppm).

Although the stomach weights of all groups of female mice given CHAH were lighter than those of the controls, there was no dose-relationship, despite a tenfold difference between the lowest and highest dose levels. Indeed, when the stomach weights were expressed relative to body weight the greatest difference from the controls was seen in the animals on the lowest dose. These observations, together with the lack of similar findings in males and the absence of any gastric lesions, suggest that the lower stomach weights in the CHAH-treated female mice were the result of unusually high control values rather than of any effect of treatment. Similarly, the isolated finding of a haemoglobin concentration 5% greater in females given 1000 ppm CHAH than in the controls is not considered to have been due to treatment. In this case it is likely that the statistical significance was the result of a small range of results in the two groups (variance ratio 4·5–5·0%) rather than a reflexion of a true difference between the two groups.

The altered ratios of lymphocytes and neutrophilic polymorphonuclear leucocytes were inconsistent, both in occurrence and in the direction of the change. Calculation of the total numbers of the different types of leucocytes showed that the alterations in the ratios were due to differing numbers of polymorphonuclear cells; a decrease in the males at wk 13 but an increase at wk 52 in females. Such calculations were not possible on the values at wk 80 since the total leucocytes were not counted. The apparently random and inconsistent nature of this change, together with the fact that the values in this experiment fell within the range seen in a concurrent experiment with the same strain of mouse (Grasso, Hardy, Gaunt, Mason & Lloyd, 1974), indicated that these alterations in leucocyte ratio are not significant in the assessment of the toxicity of CHAH.

In view of the previous suggestions that long-term administration of cyclohexylamine or the cyclamate sweeteners could lead to tumours of the urinary bladder (Price, Biava, Oser, Vogin, Steinfeld & Ley, 1970), particular attention was paid to the fixation and examination of this organ. Nevertheless, there was no evidence, from the present experiment, that CHAH treatment led to neoplastic changes in the bladder. The slightly increased incidence of a submucosal leucocyte infiltration among male mice given 1000 ppm CHAH was not reproduced at three times this dosage. In view of this lack of dose response and since the incidence in control male mice in a concurrent study (Grasso *et al.* 1974) was higher than that in the treated mice in the present experiment, it is considered that this increase represented a variation within the normal background of this finding for the strain of mouse used. The lack of adverse effect on the bladder of mice after prolonged treatment with CHAH supports the lack of effect seen in a similar study with cyclamate (Brantom, Gaunt & Grasso, 1973).

A significant finding in the short-term study on CHAH conducted in rats by Gaunt *et al.* (1974) was testicular damage in the animals given dietary levels of 2000 or 6000 ppm CHAH. Although the intakes of CHAH in the present study were similar to those in the earlier work in rats, there were no comparable effects on the testis.

The slightly increased incidences of glomerulonephrosis in the female mice given 300 ppm CHAH and of foamy macrophages in the livers of males given 1000 ppm appeared to be isolated findings since there were no indications of a dose relationship and no comparable findings in the other sex. Additionally, the frequency of these changes was within the limits encountered in a concurrent experiment (Grasso *et al.* 1974). On the other hand, the increased incidence of minor histopathological changes in the livers of the female mice at the highest level may be attributable to treatment, although the toxicological significance of this is open to doubt in view of the lack of effect in the corresponding group of males.

In no case was the frequency of occurrence of any individual tumour statistically different from the control incidence and the overall incidences of tumours were comparable in treated and control mice.

Even those tumours found without a parallel finding in the controls are considered to represent the normal incidence in mice and not to reflect any effect of CHAH. For example, the single renal adenocarcinoma in a male mouse given 3000 ppm CHAH was within the limits of the expected incidence of between 2 and 6% in this strain of mouse (Brantom *et al.* 1973). Similarly, the two renal adenomas, found only at the lowest level of treatment, represented an incidence comparable with that reported in controls from

these laboratories (Grasso *et al.* 1974) and the incidence of granulosa-cell tumours of the ovary was similar to that found in control mice of this strain (Grasso *et al.* 1974). The latter tumours have been reported by other workers (Russfield, 1967).

The two testicular tumours present in mice given 1000 ppm CHAH are uncommon (Russfield, 1967) but the spontaneous occurrence of both embryonal tumours (Stevens & Hummel, 1957) and seminoma (Gardner, 1943) has been reported. This spontaneous occurrence, together with the fact that there were no similar changes at the highest dosage level, suggests a variant of the normal distribution rather than a carcinogenic effect on this tissue. Squamous-cell carcinomas of the skin were present only in treated animals in this study but the incidence was low and without any dose relationship. Grasso *et al.* (1974) have reported this tumour in control mice of the ASH-CS1 strain and it has been encountered in other strains (Tucker & Baker, 1967).

Melanomas of the skin have been described in mice (Algire, 1944; Cloudman, 1941; Harding & Passey, 1930) and although these usually occur in coloured strains, successful transplantation to albino strains has been reported. The single melanoma encountered in this study presented as a suppurating lesion of the ear, a characteristic location for this type of tumour in mice (Cloudman, 1956).

Several sarcomas were found in treated mice, distributed randomly with no dose-related incidence. Subcutaneous sarcomas, such as those found in two male mice given 1000 and 3000 ppm CHAH and a female mouse given 300 ppm CHAH, have been reported previously in control mice of this strain (Brantom *et al.* 1973; Grasso *et al.* 1974). A diffuse spreading sarcoma apparently arising in the lungs but infiltrating the diaphragm and pancreas was found in a female mouse given 3000 ppm CHAH. Similar tumours of dubious origin, found in the peritoneal cavities of mice, have been discussed by Dobberstein & Tamaschke (1958). Two of the tumours cited by these authors were finally diagnosed as chondrosarcomas or myxochondromas by their original describers. A sarcoma of the pancreas with extensive metastases has been described by Heidenhain (1929) and sarcomas arising in spontaneous lung adenocarcinomas were mentioned by Cloudman (1941). The latter author also observed a rhabdomyosarcoma of the heart like that seen in the present study in a male mouse fed 3000 ppm CHAH. Sarcomas of various organs have been found with incidences of 0·3% (Gaunt, Mason, Grasso & Kiss, 1974), 0·4% (Brantom *et al.* 1973) and 0·6% (Grasso *et al.* 1974), while the incidence in the present study was similar at 1·3%. Therefore, although these sarcomas were found only in treated mice, the likelihood is that they represent the natural incidence of this type of tumour.

Tumours of the lymphoreticular system occur frequently in mice, and reticulum-cell neoplasms were found in all female groups and in two males fed 300 and 100 ppm CHAH, respectively. Generalized lymphosarcoma was also present in a control male and in treated males and females.

Finally the medullary tumour of the adrenal seen in a male fed 300 ppm CHAH is an example of a type of tumour that occurs spontaneously in mice (Russfield, 1967). Similar tumours were seen in a female control mouse and in one female on 300 ppm CHAH.

From the results of this experiment there is no evidence to suggest a carcinogenic action on the part of CHAH given to mice at dietary levels up to 3000 ppm for 80 wk (equivalent to an intake of approximately 400 mg/kg/day). In addition, there were no effects that could be attributed to treatment with dietary levels up to 1000 ppm (equivalent to an intake of approximately 140 mg/kg/day) and this level can be considered as the no-untoward-effect level. At the higher level of 3000 ppm there were hepatic changes of doubtful significance and the true no-effect-level may be nearer this figure. In terms of the cyclamate sweeteners, this study has failed to detect any carcinogenic effect from the administration to mice of a dose of CHAH over ten times that which would be produced by a quantitative conversion of the maximum recommended dose of cyclamate (Joint FAO/WHO Expert Committee on Food Additives, 1968).

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EFFECTS OF BUTYLATED HYDROXYANISOLE ON *TETRAHYMENA PYRIFORMIS**

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Abstract—The biological effect of butylated hydroxyanisole (BHA) at the molecular level in a eukaryotic system was studied using *Tetrahymena pyriformis*. BHA at 20 ppm in the medium inhibited cell growth by 50% and the synthesis of DNA, RNA and protein also by 50%. Kinetic experiments indicated that inhibition of the synthesis of all three types of macromolecule occurred simultaneously, within 10 min of the addition of the BHA to the media. No effect was observed on other forms of macromolecular synthesis, and the cell membrane did not appear to be affected. Cells exposed to 20 ppm BHA had a normal morphology and size distribution.

INTRODUCTION

Butylated hydroxyanisole (BHA) is a phenolic antioxidant used as a food additive to prevent the autoxidation of unsaturated lipids. The acute toxicity of BHA is low, the LD_{50} in most animals being greater than 2 g/kg body weight (Hathway, 1966). However, there is a major concern about the long-term toxicity of this antioxidant. When administered to rats at 500 mg/kg body weight, BHA caused moderate increases in liver weight (Allen & Engblom, 1972). However, the compound is considered to be non-hepatotoxic because the liver changes are reversible when it is removed from the ration (Johnson, 1971).

Recently, Allen & Engblom (1972) reported that when BHA was administered to rhesus monkeys at 500 mg/kg body weight, nucleolar fragmentation occurred and large intranucleolar fibrils were present in many of the hepatocytes. Also, Branen, Richardson, Goel & Allen (1973) reported that BHA prevented some of the lipid changes elicited by corn oil used as solvent for the antioxidants in the same animals; BHA lowered the levels of liver cholesterol, liver phospholipid, liver triglycerides and plasma total cholesterol.

The diverse morphological and biochemical effects of BHA in animals have not been satisfactorily explained, and it seemed appropriate to study the potential toxicity of this antioxidant in a more simplified system. The ciliated protozoan, *Tetrahymena pyriformis*, offers distinct advantages for such a study. It is a single-cell organism constituting homogeneous culture, easily grown in an axenic medium, and has nutritional requirements similar to those of higher animals (Hill, 1972). The following report describes a study designed to determine the mechanism of action of BHA at the cellular level, using Tetrahymena as a model test system.

EXPERIMENTAL

Materials. Chemicals were obtained from the following sources: proteose peptone and yeast extract from Difco Laboratories, Detroit, Mich., BHA (food grade) from Eastman Chemical Products, Rochester, N.Y., dimethylsulphoxide (DMSO) from Sigma Chemical Co., St. Louis, Mo., Soluene 100 from Packard Instrument Co., Chicago, Ill., [*Me*-^{3}H]thymidine and [5-^{3}H]uridine from New England Nuclear, Boston, Mass., and D-[1-^{14}C]glucose from Calbiochem, San Diego, Cal. All other reagents used were of the highest grade obtainable commercially.

Growth measurements of Tetrahymena. T. pyriformis type E was incubated without shaking in the dark at 24 ± 1°C in 1 litre flasks filled with 200 ml autoclaved medium containing 2% (w/v) proteose peptone and 0·1% (w/v) yeast extract. For experimental purposes, a 1% inoculum of early log growth cells was used. BHA, as a concentrated solution dissolved in DMSO, was added at the time of inoculation or to an 18-hr culture. Growth of *T. pyriformis* was measured by drying a washed aliquot of cells to constant weight, or counting the numbers of cells with a Coulter Model B Counter previously calibrated with ragweed pollen using a 200 μm orifice. For counting, 0·5 ml of cell suspension was added to a 0·85% (w/v) NaCl solution containing 0·2% (w/v) formaldehyde. Size distribution was determined by setting upper and lower threshold limits at discrete intervals.

Radioactivity measurements. Following incubation of the culture with the appropriate radioisotope for a designated time, the cells were harvested by centrifugation (600 **g** for 5 min) and washed three times

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with cold deionized water. The procedure of Shrug, Elson & Shrago (1969) was used to determine the amount of [^{14}C]acetate incorporated into protein, lipid and glycogen, and that of Milner (1967) to determine the incorporation of [^{14}C]amino acid hydrolysate into protein, [^{3}H]thymidine into DNA and [^{3}H]uridine into RNA. Kinetics of protein, RNA and DNA synthesis and transport of D-[1-^{14}C]glucose were measured by pre-incubating the radioisotope for 1·5 hr before the addition of BHA. At predetermined time intervals, aliquots of cells were removed, cooled, harvested by sedimentation (100 g at 30 sec), washed three times with cold deionized water and transferred to scintillation vials, in which the cells were lysed with 12 ml of scintillation fluid (Bray, 1960).

Radioactivity was measured by adding 12 ml of Bray's scintillation fluid to the vials and counting the radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrometer calibrated for maximum counting. When a precipitate was present, the vials were shaken prior to measurement. Quenching was corrected by the use of automatic external standardization.

Light-microscopic observations. BHA was added to a suspension of cells and allowed to pre-incubate for 2 min. An aliquot of 0·1 ml was then removed for viewing for 18 min through a Zeiss research microscope equipped with a 50× phase contrast ocular.

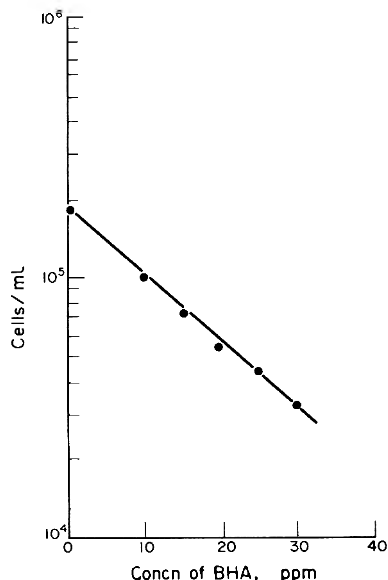

Fig. 2. Cell concentration of *T. pyriformis* after incubation for 8 hr with BHA, added as a concentrated solution in DMSO to an early log growth culture. Cell growth was measured by counting cell number using a Coulter Model B Counter.

RESULTS

Growth

BHA inhibited the growth of *T. pyriformis* when added to an 18-hr culture which was in early log growth (Fig. 1). At the concentration used, DMSO had no effect on cell growth when added alone to a culture. Within 18 hr of the addition of BHA to the media, there was a measurable dose-dependent decrease in the population density of the cells (Fig. 2) and increase in the generation time (Table 1). There was also a decrease in cell number in the stationary growth phase compared with cultures without added BHA.

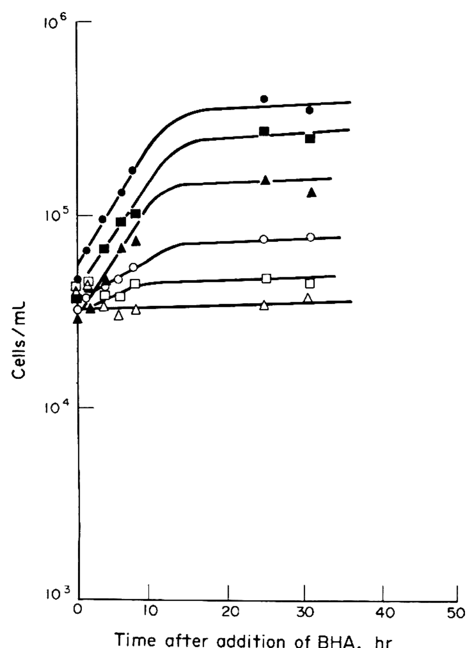

Fig. 1. Growth curves of *T. pyriformis* following addition of BHA as a concentrated solution in DMSO to an early log growth culture. Cell growth was measured by counting cell number using a Coulter Model B Counter, and concentrations of BHA in the media were 0 (●), 10 (■), 15 (▲), 20 (○), 25 (□) and 30 (△) ppm.

Size and shape

BHA was added to the medium at a final concentration of 20 ppm and the cells were examined 20 min later. There was no effect on the gross morphology of *T. pyriformis* when viewed through the phase contrast microscope. The cells were a normal pyriform shape and the cilia beat normally. Similar results were observed when the cells were exposed to 60 ppm BHA for 20 min. Furthermore, when measured by the Coulter Counter, the cells had a normal relative size distribution after exposure to 0–20 ppm BHA for 1·5 hr.

Table 1. *Generation times of* T. pyriformis *in media containing BHA*

Concn of BHA* (ppm)	Generation Time (hr)
0	3·34
10	3·55
15	4·74
20	4·93
25	10·10
30	—

*BHA as a concentrated solution in DMSO was added to an early log growth culture of *T. pyriformis*.

Values were calculated as the slope of the growth curves from Fig. 1.

Table 2. *The effect of BHA on incorporation of* [^{14}C]-*acetate into lipids, protein and glycogen of* T. pyriformis

Concn of BHA* (ppm)	Radioactivity (dpm/mg dry weight)			
	Total	In lipid	In protein	In glycogen
	Early log phase culture			
0	8789	6288	979	92
10	10015	7158	731	63
20	6829	5378	375	158
	Stationary growth phase culture			
0	6143	580	830	1505
10	6110	601	818	1317
15	6943	698	873	1653
20	6897	701	863	1560

*Final concentration of BHA in the culture after addition of BHA as a concentrated solution.

Values are the amounts of total radioactivity and of radioactivity incorporated into purified fractions of lipid, protein and glycogen when cultures of *T. pyriformis* in either log growth phase or stationary growth phase were incubated for 1 hr with 0·5 μCi sodium [2-^{14}C]acetate. Values were corrected for quenching by automatic external standardization.

Radioisotope measurements

BHA added to growing or stationary cultures had no effect on the incorporation of [^{14}C]acetate into lipids and glycogen (Table 2). However, during early log growth there appeared to be a slight decrease in protein synthesis compared with the control. Addition of BHA to the growth medium markedly inhibited the incorporation of [^{14}C]amino acids into protein,

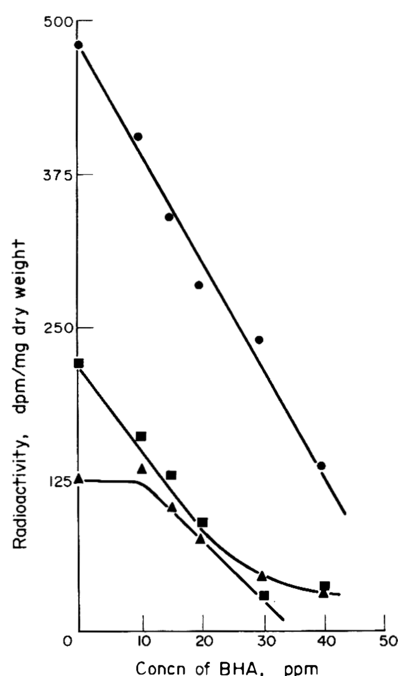

Fig. 3. Effect of various concentrations of BHA, added as a concentrated solution in DMSO to an early log growth culture of *T. pyriformis*, on the synthesis of DNA (▲), RNA (■) and protein (●), determined after incubation for 3 hr with [^{3}H]thymidine (1·0 μCi), [^{3}H]uridine (1·0 μCi) and a ^{14}C-labelled amino acid hydrolysate (0·5 μCi), respectively. Quenching was corrected by AES.

of [^{3}H]uridine into RNA and of [^{3}H]thymidine into DNA (Fig. 3).

Kinetic experiments were performed to determine the mechanism and time sequence of inhibition by BHA. Inhibition of synthesis of protein (Fig. 4a), of RNA (Fig. 4b), and of DNA (Fig. 4c) developed within 10 min of addition of BHA. DNA synthesis was completely inhibited for 1·5 hr after which a partial recovery was evident. From these results, it is impossible to determine whether the primary effect was on DNA, RNA or protein synthesis, since under the experimental conditions used inhibition of all three macromolecules occurred simultaneously.

BHA had only a very small inhibitory effect on glucose transport in cultures of *T. pyriformis* (Fig. 4d).

DISCUSSION

T. pyriformis is a useful organism for studying the direct effect of chemicals on enzyme and membrane systems. It is a eukaryotic cell that contains all of the mammalian organelles, has metabolic pathways similar to those of mammals, has complex nutritive requirements, and is easily grown in culture. Several antibiotics and other drugs studied in *T. pyriformis* have effects similar to those demonstrated in other living systems (Hill, 1970).

BHA, when added to a culture of *T. pyriformis*, caused a dose-dependent inhibition of cell growth. No changes were observed in morphology or in the size distribution of the cells.

The primary effect of BHA on *T. pyriformis* appeared to be at the level of DNA, RNA and protein synthesis (Fig. 3), the extent of inhibition being dependent on concentration. With 20 ppm BHA in the medium, there was a 50% inhibition of DNA, RNA and protein synthesis. The lack of effect on the synthesis of either lipids or glycogen from [^{14}C]acetate when BHA was added to an early log phase culture (Table 2), indicated that BHA had no specific effect on intermediary metabolism in *T. pyriformis*. BHA did not inhibit [^{14}C]acetate incorporation into protein, lipids or glycogen in stationary growth phase cells (Table 2), an expected finding since the general metabolic rates of the organism would normally be depressed under these circumstances.

BHA did not appear to have any effect on the cell membrane of *T. pyriformis*. The slight inhibition of glucose transport may have been due to a lower glucose requirement secondary to a decrease in the overall growth rate. When viewed under the phase-contrast microscope, the cells appeared to have a normally functioning water-expulsion vacuole. This vacuole functions by excreting excess water, enabling the organism to maintain homeostasis.

A series of kinetic experiments was performed in order to determine the sequential inhibition of DNA, RNA and protein synthesis by BHA. The results showed that inhibition of DNA, RNA and protein synthesis occurred simultaneously, within 10 min of the addition of 20 ppm BHA (Figs 4a–c). If only DNA synthesis had been inhibited, protein and RNA synthesis would have continued for at least 10 min following the addition of the drug (Jeffery, 1974). Specific inhibition of DNA synthesis would also have

caused an increase in cell size and a synchronization of cell division. Colchicine, a drug that specifically inhibits DNA synthesis, delays the S phase of the cell cycle, causing synchronization of cell division (Hill, 1972). Moreover, the synthesis of RNA and protein would have continued, with an increase in cell size. Neither of these two phenomena was observed when 20 ppm BHA was added to an early log growth culture of *T. pyriformis*. When specific inhibition of RNA synthesis occurs, DNA and protein synthesis continue at normal rates for at least 10 min (Jeffery, 1974). No continuation of the synthesis of either DNA, RNA or protein was observed after BHA was added to a culture of *T. pyriformis*.

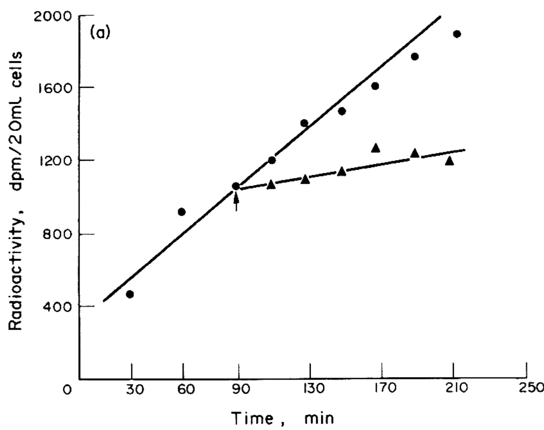

Fig. 4(a)

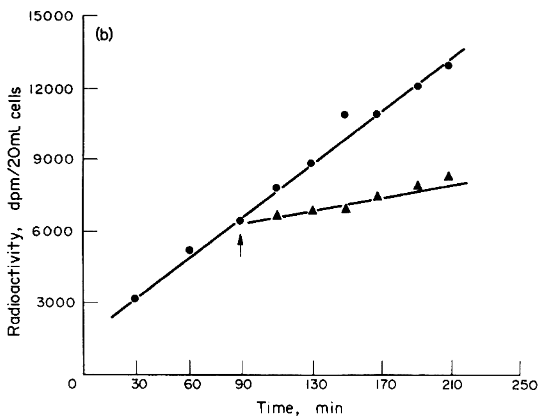

Fig. 4(b)

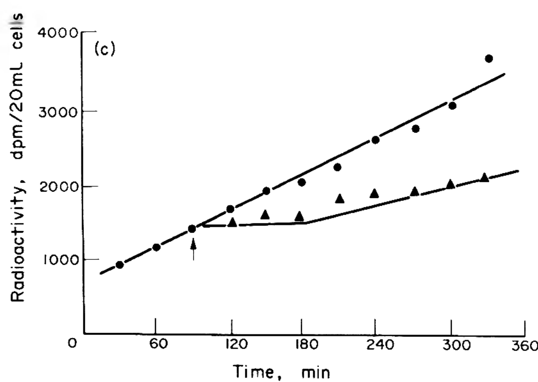

Fig. 4(c)

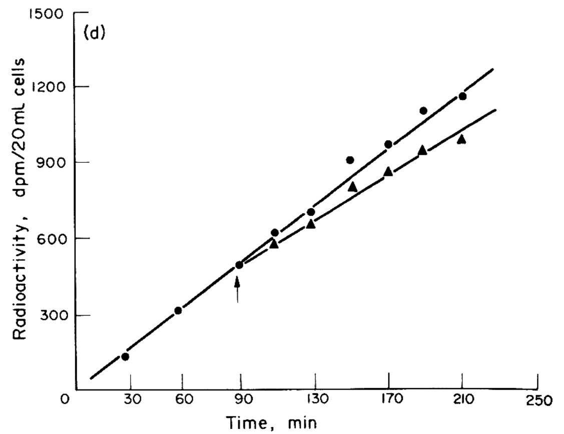

Fig. 4(d)

Figs. 4 (a)–(d). The effect of 0 (●) and 20 (▲) ppm BHA on the kinetics of (a) protein synthesis, (b) RNA synthesis, (c) DNA synthesis and (d) glucose transport in *T. pyriformis* cultures containing (a) ^{14}C-labelled amino acid hydrolysate (1·0 μCi), (b) [^{3}H]uridine (5·0 μCi), (c) [^{3}H]thymidine (5·0 μCi) and (d) [1-^{14}C]glucose (1·0 μCi). The time of addition of the concentrated solution of BHA in DMSO to the culture medium is arrowed. Quenching was corrected by automatic external standardization.

BHA has been shown to bind to the polypeptide, bradykinin (Donaldson, 1973; Posati & Pallansch, 1970). This might suggest a possible linking to proteins or nucleoproteins in *T. pyriformis* causing growth inhibition. Allen & Engblom (1972) reported nucleolar fragmentation and the presence of large intranuclear fibrils in many hepatic nuclei when juvenile rhesus monkeys were given BHA in a dose of 500 mg/kg body weight. However, there was no significant decrease in the amount of DNA, RNA or total protein in the livers of the BHA-treated monkeys (Allen & Engblom, 1972). These changes have been associated with a disruption of nucleolar function. Additional experiments are necessary to determine more specifically the site of action of BHA in *T. pyriformis*. It is premature to extrapolate the results from this study to mammalian systems, but experiments are currently being conducted to determine whether similar results will be obtained when hepatocytes are studied *in vitro*.

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A COMPARISON OF THE DISTRIBUTION AND ELIMINATION OF OLEIC AND CHLORINATED OLEIC ACIDS AND THEIR METABOLITES IN RATS

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Abstract—Twenty-four 100-g rats were given [^{3}H]oleic and ^{36}Cl-chlorinated oleic acid by intubation and killed at intervals from day 1 to day 28. Faeces and urine were collected from four rats for 28 days. The chlorinated oleic acid was 72·3% absorbed compared to 91·5% of the non-chlorinated acid. The brain was observed to discriminate more against the uptake of the chlorinated lipid than of the non-chlorinated lipid, but little discrimination was observed in other tissues, and mobilization of ^{36}Cl from lipids of adipose and kidney tissue was similar to that of ^{3}H. The liver appeared to be the main site for dechlorination of the chlorinated fatty acids. Chlorinated lipids had a half-life of 19·5 days in kidney, 10·7 in liver, 10·0 in brain, 8·3 in heart and 5·1 in blood.

INTRODUCTION

Chlorine gas is commonly used as a bleaching agent in cake flour, and recent studies have shown that levels used commercially produce a flour that may have significant effects in rats when administered as 87% of their diet (H. M. Cunningham, G. A. Lawrence and L. Tryphonas, unpublished data 1976). The most common sign observed in rats fed diets containing chlorinated flour was a reduction in growth rate. Both chlorinated flour lipids and chlorinated wheat gluten were found to have adverse effects, but the degree to which chlorinated wheat starch may affect rats has yet to be determined. Since the chlorinated flour lipids appeared to be slightly more toxic than the chlorinated gluten, the metabolism of chlorinated lipids was studied first.

Preliminary studies indicated that the chlorination of flour lipids produced a variety of unidentified compounds more polar than the original lipids. Since oleic acid is a major fatty acid in wheat lipids (Hilditch, 1956) and traces of dichlorostearic acid have been detected in lipids from chlorine-treated flour (Daniels, Frape, Russell Eggitt & Coppock, 1963), it was decided that the first metabolism studies should be conducted with chlorinated oleic acid.

The metabolism of glycerol mono-oleate chlorinated with ^{38}Cl prepared by neutron activation has been studied in pigs, but the radioactive half-life of 37·5 min did not permit measurements on the biological half-life. Since no other labelled form of oleic acid was used for comparison, the authors had no means of verifying their claim that the chlorinated oleic acid was metabolized by normal metabolic pathways (Sink, Watkins, Ziegler & Kean, 1965).

Chlorine-36 has a half-life of $3{\cdot}1 \times 10^5$ yr and was successfully used in earlier work to measure the distribution of chlorine in chlorinated flour (Cunningham *et al.* 1976). The present study was designed to compare the metabolism, distribution and biological half-life of oleic acid chlorinated with ^{36}Cl with that of [^{3}H]oleic acid in rats.

EXPERIMENTAL

Materials. Chlorine-36 was purchased as 3·25 N-H^{36}Cl with a specific activity of 4·4 mCi/g from Atomic Energy of Canada Limited, Commercial Products, Ottawa. Oleic acid was obtained from Serdary Research Laboratories Inc., London, Ontario, and [9,10-^{3}H(N)]oleic acid (5·71 Ci/mmole) from New England Nuclear, Boston, Mass., USA. Chlorine was generated by the reaction of HCl and $KMnO_4$ (Holmes, 1941). Oleic acid (1 g) was placed in a 250-ml round-bottomed flask with a side arm containing 0·5 g $KMnO_4$, the system was evacuated and 0·528 ml conc. HCl and 100 μCi H^{36}Cl were carefully added dropwise to the side arm. Although production of chlorine was immediate, several hours were allowed for it to react with the oleic acid. The theoretical yield of chlorine from HCl was 62·5%, and enough carrier HCl was used to produce, overall, approximately 15% chlorinated oleic acid. The chlorinated oleic acid was placed on a rotary evaporator at 70°C for 30 min to remove any free chlorine or HCl and then 2·0 mCi [^{3}H]oleic acid was introduced, and enough corn oil, as a vehicle, was added to bring the volume up to 40 ml. Thin-layer chromatography indicated that 85% of the ^{36}Cl was present as dichlorostearic acid and the remainder as more polar unidentified compounds.

Animals and administration. Twenty-four 100-g male Wistar rats were each given by intubation 1·0 ml of the doubly labelled preparation of oleic acid in corn oil, and groups of four animals were killed at 1, 2, 3, 7, 14 and 28 days after administration. The last group were placed in metabolism cages immediately after dosing, and urine and faeces were collected for 28 days. All rats were fed a standard laboratory rat chow diet.

Analytical methods. Samples of blood, liver, kidney, heart, omentum, brain and faeces were analysed in duplicate for total lipids by Folch extraction (Folch, Lees & Sloane Stanley, 1957). The lipids were dis-

solved in Aquasol® and the activities of ^3H and ^{36}Cl were counted on a Beckman Model LS-230 liquid scintillation counter using an external standard for quench correction. Samples of the aqueous phase from the Folch extraction and samples of urine were also dissolved in Aquasol and counted.

To determine whether any ^{36}Cl was incorporated into tissue protein, the protein residue from the Folch extraction was purified of lipid and of water-soluble materials by drawing off the residual solvents and extracting the residue twice with chloroform–methanol–water (2:1:1, by vol.), once with methanol–diethyl ether (2:1, v/v) and once with diethyl ether. The residue was dried at room temperature and 10-mg samples were homogenized with 1·0 ml H_2O and a few ml of Aquasol. To ensure that no chlorine remained loosely bound to the residue, aliquots were dissolved in dilute NaOH, precipitated with HCl and trichloroacetic acid, centrifuged to remove the salt and acid solution and washed twice with 5% trichloroacetic acid. The residue was washed once with methanol–diethyl ether (2:1) and once with diethyl ether, and 10-mg samples were again homogenized with 1 ml H_2O and Aquasol and counted.

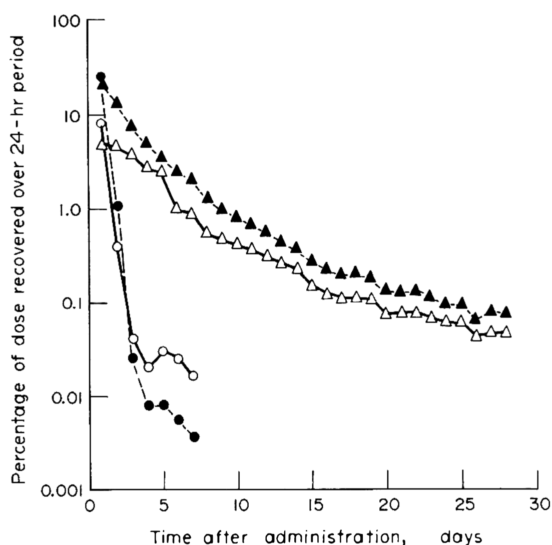

Fig. 1. Excretion of ^3H (△, ○) and ^{36}Cl (▲, ●) in the faecal lipids (○, ●) and urine (△, ▲) of rats after intubation with 25 mg of 15%-chlorinated oleic acid containing 50 μCi [^3H]oleic and 1·5 μCi [^{36}Cl]dichlorostearic acid.

RESULTS

The rats excreted 8·69 ± 2·78% of the dose of ^3H and 27·61 ± 4·96% of the ^{36}Cl in the faecal lipids during the first 7 days after dosing (Fig. 1). Thereafter, the amount present became too low to determine with accuracy. The ^3H and ^{36}Cl in the aqueous fraction of faeces (not shown in Fig. 1) represented 4·71 ± 0·42 and 3·82 ± 0·30% of the dose, respectively, during the same period. A large percentage of the dose of ^{36}Cl appeared in the urine during the first few days compared to only small amounts of ^3H (Fig. 1). The total 28-day urine excretion amounted to 17·51% for ^3H and 59·79% for ^{36}Cl. The total recovery of the administered radioactivity in the urine and faeces amounted to 30·91 ± 2·2% of the ^3H and 91·32 ± 3·34% of the ^{36}Cl. The amount of ^3H and ^{36}Cl excreted in the urine from day 15 to day 28 (Fig. 1) decreased almost linearly, the half-life calculated from this portion of the curve being 8·0 days for both tracers.

The tissue-water concentration of ^{36}Cl (Fig. 2) was lower than that of ^3H in the liver, heart and brain throughout the experiment, with a tendency for the difference to increase with time. Only in the tissue water of the blood, kidney and omentum was the concentration of ^{36}Cl initially higher than that of ^3H, after which the order reversed and the difference again increased.

Highest concentrations of ^{36}Cl-labelled lipids in rats were found in the omentum followed by the kidney, heart, liver, brain and blood (Fig. 2). The heart was the only tissue in which the initial 24-hr lipid ^{36}Cl was higher than that of the ^3H. The brain lipids did not take up much of either label, discrimination against the ^{36}Cl lipids being about four times greater than that against the ^3H lipids. In the kidney and omentum the ratio of ^{36}Cl to ^3H lipids did not change appreciably during the experiment, but in the brain, blood, liver and heart the ratio decreased as the experiment progressed, the lowest ratio of ^{36}Cl to ^3H (1:33) being found in the brain at day 24. The concentration of both ^{36}Cl- and ^3H-labelled lipids increased in the omentum from wk 2 to wk 4 owing to a 3·4-fold increase in the lipid content of the omentum concurrent with the decline in actual lipid concentration of the labels during this period (Fig. 3). The perirenal fat depots also increased considerably during this period and a spot check on the lipid concentration of ^{36}Cl in the perirenal fat of one rat on day 28 indicated that it was 82% of that in the omentum.

Calculation of the total accumulated radioactivity in each tissue (Table 1) has the advantage over calculation of the concentration in tissues (Fig. 2) that differences with time are unaffected by dilution as the tissue enlarges. Highest initial accumulation of ^{36}Cl was observed in the lipids of the liver, followed in decreasing order by the omentum, kidney, heart, blood and brain. The terminal portions of the curves were more linear than those in Fig. 2, so the biological half-life was calculated (Table 1) for this part of the curve. The longest half-life of the non-chlorinated lipids was observed in the brain, followed by the kidney, heart, blood and liver. The half-life of the chlorinated lipids was less than that of the non-chlorinated lipids in the corresponding tissues, the greatest differences being found in the brain, followed by the blood, heart, kidney and liver. The biological half-life of the omental lipids could not be calculated owing to the excess accumulation of both labelled and non-labelled lipids during the last 2 wk of the experiment.

An average of 0·0817 ± 0·007% of the dose of ^{36}Cl was detected in the liver proteins 24 hr after dosing but none was detected thereafter. None of this ^{36}Cl was removed by NaOH treatment and none of the label was detected with certainty in the protein of any other tissue.

®Registered trade name of New England Nuclear, Boston, Mass.

Table 1. *Uptake and biological half-life of oleic and of dichlorostearic acid in the tissues of rats intubated with 25 mg of 15% chlorinated oleic acid containing 50 μCi [^{3}H]oleic and 1·5 μCi [^{36}Cl]dichlorostearic acid*

Tissue	Isotope	Uptake of radioactivity (percentage of dose)* at day						Half-life (days)§
		1	2	3	7	14	28	
Liver	^{3}H	0·5102 ± 0·1066	0·2922 ± 0·0140	0·1425 ± 0·0140	0·0490 ± 0·0010	0·0445 ± 0·0055	0·0182 ± 0·0041	12·6
	^{36}Cl	0·3571 ± 0·0685	0·1450 ± 0·0229	0·0529 ± 0·0118	0·0115 ± 0·0010	0·0075 ± 0·0009	0·0030 ± 0·0004	10·7
Kidney	^{3}H	0·2210 ± 0·0101	0·1605 ± 0·0120	0·0845 ± 0·0084	0·0497 ± 0·0097	0·0343 ± 0·0052	0·0233 ± 0·0052	23·6
	^{36}Cl	0·1057 ± 0·0081	0·0780 ± 0·0071	0·0352 ± 0·0070	0·0217 ± 0·0052	0·0172 ± 0·0025	0·0102 ± 0·0023	19·5
Heart	^{3}H	0·0257 ± 0·0016	0·0178 ± 0·0020	0·0105 ± 0·0006	0·0060 ± 0·0008	0·0037 ± 0·0001	0·0021 ± 0·0003	15·0
	^{36}Cl	0·0284 ± 0·0028	0·0148 ± 0·0019	0·0077 ± 0·0004	0·0032 ± 0·0001	0·0012 ± 0·0005	0·0008 ± 0·0002	8·3
Brain	^{3}H	0·0198 ± 0·0004	0·0220 ± 0·0014	0·0206 ± 0·0008	0·0167 ± 0·0005	0·0139 ± 0·0003	0·0083 ± 0·0001	23·8
	^{36}Cl	0·0055 ± 0·0004	0·0032 ± 0·0006	0·0018 ± 0·0001	0·0010 ± 0·0001	0·0007 ± 0·0002	0·0002 ± 0·0001	10·0
Omentum†	^{3}H	0·5292 ± 0·0401	0·4887 ± 0·0910	0·3230 ± 0·0642	0·1957 ± 0·0641	0·1170 ± 0·0426	0·2850 ± 0·0646	
	^{36}Cl	0·3525 ± 0·0413	0·3640 ± 0·0211	0·2217 ± 0·0412	0·1430 ± 0·0431	0·0896 ± 0·0149	0·1521 ± 0·0287	
Blood‡	^{3}H	0·0447 ± 0·0070	0·0250 ± 0·0015	0·0183 ± 0·0009	0·0120 ± 0·0010	0·0053 ± 0·0002	0·0032 ± 0·0009	13·1
	^{36}Cl	0·0283 ± 0·0039	0·0113 ± 0·0012	0·0065 ± 0·0006	0·0024 ± 0·0002	0·0007 ± 0·0001	0·0001 ± 0·0002	5·1

*Values are means ± SEM for groups of four animals.
†The omentum was not totally recovered and data are given as % of dose recovered/g omentum.
‡The total blood volume was calculated as 6·41% of body weight (Altman & Dittmer 1974).
§The biological half-life was obtained from the line best fitting the terminal linear portion of the curve of the data plotted on a semilogarithmic scale.

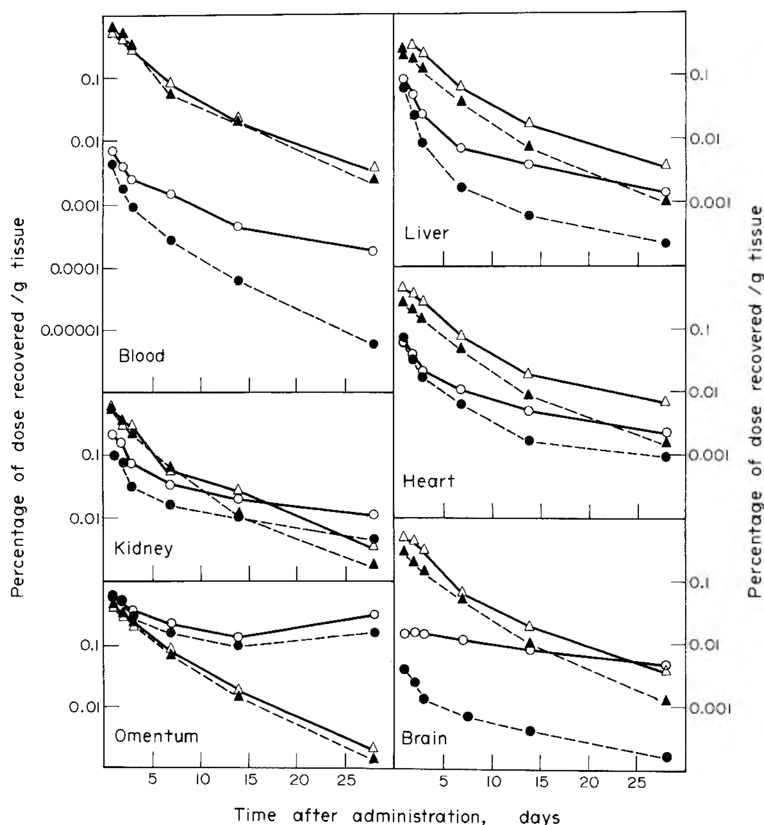

Fig. 2. Distribution of ^{3}H (△, ○) and ^{36}Cl (▲, ●) in tissue water (△, ▲) and in tissue lipids (○, ●) of rats after intubation with 25 mg of 15%-chlorinated oleic acid containing 50 μCi [^{3}H]oleic and 1·5 μCi [^{36}Cl]dichlorostearic acid. Values are reported for unit weight of tissue.

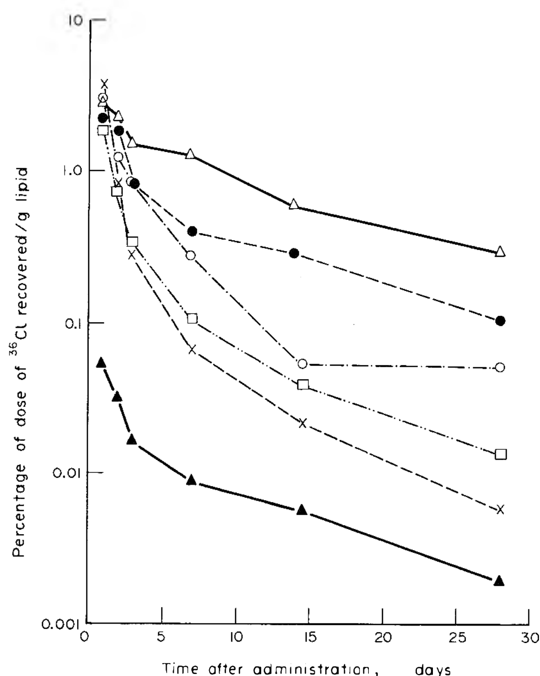

Fig. 3. Concentration of ^{36}Cl in the lipids of the omentum (△), kidney (●), heart (○), blood (□), liver (×) and brain (▲) of rats after intubation with 25 mg of 15%-chlorinated oleic acid containing 1·5 μCi [^{36}Cl]dichlorostearic acid. Values are reported for unit weight of lipid.

DISCUSSION

The data show that there are a number of mechanisms by which the body discriminates against chlorinated fatty acids. First, as judged by the excretion of radioactivity in the faecal lipids, only 72·3% of the chlorinated oleic acid was absorbed in the digestive process compared to 91·5% of the non-chlorinated acid. The degree to which this discrimination is caused by the saturated nature of the chlorinated oleic acid (dichlorostearic acid) compared to the unsaturated tritiated oleic acid is difficult to determine, but, since the chlorinated oleic acid was accompanied by 39 volumes of corn oil, a large reduction in digestibility would not be expected (Cunningham & Leat, 1969; Morehouse, Skipski, Searcy & Spolter, 1956).

The recovery of only 31% of the ^{3}H in the faeces and urine as against 91% of the ^{36}Cl can probably be attributed to expiration of ^{3}H in insensible water (Cunningham, 1968). Only a small amount of the difference could be accounted for by the higher level of tritiated lipids remaining in the body of the rats at day 28. There was also the possibility of recycling of the ^{3}H label by metabolism to water and reincorporation into lipids, but the degree to which this occurred in the present studies was believed to be small, since in earlier work only 3% of an iv dose of tritiated water in pigs was incorporated into carcass lipids over a period of 2 wk (Cunningham, 1968).

Once the ^{36}Cl was split off from the lipids and had entered the tissue water, it tended to move with the ^{3}H of the body water and at similar levels in all tissues, as would be expected for chloride salts. The kidneys, which are the terminal organs in the excretion of chloride, appeared to maintain the highest concentration of ^{36}Cl in tissue water.

The present work demonstrates that the brain has a more efficient barrier against chlorinated fatty acids than that normally observed with unchlorinated fatty acids (Dhopeshwarkar & Mead, 1973). The brain incorporated not only much less ^{3}H in its lipids than did other tissues but only a quarter as much ^{36}Cl as ^{3}H. This discrimination continued throughout the experiment, the concentration of ^{36}Cl in the brain lipids running parallel to, but much below, that in the blood (Fig. 3).

The brain was capable of discriminating against chlorinated lipids from the beginning of the experiment, and since the decline of ^{36}Cl in the brain paralleled that in the blood, this decline did not necessarily indicate an ability to metabolize or dechlorinate chlorinated fatty acids. The liver, which had the highest lipid concentration of ^{36}Cl at 24 hr was the only organ apart from the brain to reduce this level eventually to below that of the blood (Fig. 3). The high levels of non-lipid ^{36}Cl excreted in the urine during the first few days indicated that a dechlorinating mechanism was functioning in the body. The liver was the only tissue in the body in which a significant amount of ^{36}Cl was detected bound to protein. This suggests a possible intermediate in the metabolism of chlorinated lipids and provides further evidence that the liver is a likely site for their dechlorination.

Whether or not dechlorination of lipids can occur in other tissues of the body cannot be determined from the present experiment. The concentration of ^{36}Cl in the heart lipids also declined during the experiment but not to the same extent as in the blood and could simply have been the result of an exchange of lipids with those in the blood rather than dechlorination. On the other hand, the chlorinated fatty acids could be carried at a higher concentration in some fractions of the blood than in others, such as the free fatty acids (FFA) of the plasma. This fraction represents only 1–5% of the plasma lipids but has a rapid turnover and can account for most of the net lipid transport in the blood (Fredrickson & Gordon, 1958). Therefore, the plasma FFA could have a very high concentration of ^{36}Cl relative to that of whole blood and contribute to an increase in the ^{36}Cl concentration in the lipids of tissues to give levels that are higher than the average concentration in whole-blood lipids.

This could also explain how lipids containing a high concentration of ^{36}Cl were apparently moved from some areas in the body into adipose tissue, while low concentrations were continuously being recorded in the total blood lipids. The 3·4-fold increase in the lipid content of the omentum was accompanied by only a 50% decline in the omental-lipid concentration of ^{36}Cl. This is less than would be expected to occur if the new lipids contained no chlorine, and indicates that chlorinated lipids were redistributed in the body, a large proportion appearing in the adipose tissue. The fact that just as much ^{3}H-labelled lipid (Fig. 2) was deposited in the omentum at this time means that the ^{36}Cl-labelled lipids were not selectively removed from other storage sites to be shunted into the omental adipose tissue.

The increase in omental lipids during the last 2 wk of the experiment made it difficult to estimate the biological half-life of chlorinated lipids in adipose tissue. The kidney lipids had the longest half-life of any organ lipids examined (23·6 days for ^{3}H and 19·5 days for ^{36}Cl; Table 1). When determined as a percentage of the dose recovered/g lipid, half-life values of 11·0 and 9·3 days for ^{3}H and ^{36}Cl, respectively, were obtained in kidney lipids, compared to 12·7 and 10·7 days, respectively, for omental lipids. The half-life of chlorinated lipids in adipose tissue is therefore similar to that of non-chlorinated lipids. The reported values for the half-life of carcass fat in rats vary between 16 and 141 days (Gorin & Shafrir, 1963; Jansen, Hutchison & Zanetti, 1966; Phil, Bloch & Anker, 1950; Thompson & Ballou, 1956). When one considers that the half-life of fatty acids in human adipose tissue may range from 350 to 700 days (Fleischman, Hayton, Bierenbaum & Watson, 1968; Hirsch, Farquhar, Ahrens, Peterson & Stoffel, 1960) it may be realized that the consumption of chlorinated cake flour could result in a prolonged residue of chlorinated lipids in the body.

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FREQUENCY OF POLYPLOID CELLS IN THE BONE MARROW OF RATS FED IRRADIATED WHEAT

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Abstract—Diets containing different proportions of non-irradiated or irradiated wheat were fed to Wistar rats for 1 or 6 wk. Cytological analysis of the bone marrow showed no significant difference in the frequency of polyploid cells in the rats fed non-irradiated or irradiated wheat diets, even when the treated wheat was fed to the rats within 24 hr of irradiation.

INTRODUCTION

The Joint FAO/IAEA/WHO Expert Committee (1970), which reviewed data relating to the wholesomeness of irradiated wheat, stated that extensive studies in animals fed wheat irradiated with up to 200 krad had revealed no evidence that irradiated wheat was toxic or carcinogenic. However, in view of changing practices in the toxicological evaluation of all chemicals, including drugs, pesticides, food additives and air and water pollutants, the Committee also recognized the need for studies involving the *in vivo* evaluation of irradiated foods for mutagenicity and cytotoxicity in mammals. Meanwhile, with the exception of Moutschen-Dahmen, Moutschen & Ehrenberg (1970), who reported an increase in preimplantation losses in mice after the feeding of an irradiated diet, investigators (Chauhan, Aravindakshan, Aiyar & Sundaram, 1975a,b; Eriksen & Emborg, 1972; Renner, Grünewald & Ehrenberg-Kieckebusch, 1973) have observed no evidence of induced mutagenicity in tests for dominant lethals in rats and mice fed diets exposed to high doses of radiation (2·5–5 Mrad) and containing wheat as a major component. Similarly, the feeding of freshly irradiated wheat (75 krad) did not induce any mutagenicity in male Wistar rats (Chauhan, Aravindakshan, Kumar, Subba Rao, Aiyar & Sundaram, 1976).

Recently, wheat irradiated at 75 krad and fed within 20 days of irradiation has been reported to induce a high frequency of polyploid cells in the bone marrow of rats (Vijayalaxmi & Sadasivan, 1975). Since an effect of this kind has never been suggested as a consequence of the consumption of irradiated diet, it was considered of interest to examine these observations. Our findings on the frequency of polyploid cells in the bone marrow of rats fed irradiated wheat are reported in this communication.

EXPERIMENTAL

Animals and diets

Female inbred Wistar rats reared at the Centre were used throughout the study.

Experiment 1. Rats, 6–8 wk old, were randomly assigned to two groups of six animals and fed for 8 wk on a stock diet (wheat flour 70%, Bengal gram (*Cicer arietinum*) 20%, yeast powder 4%, fish meal 5% and sesame oil supplemented with shark liver oil 1%) containing non-irradiated or irradiated wheat (control and test groups, respectively). In accordance with the established procedure for testing the wholesomeness of irradiated foods, the irradiated wheat was stored for 2 wk at room temperature, along with control samples, before being powdered for incorporation in the diet.

Experiment 2. Rats, again 6–8 wk old, were randomly divided into ten groups of six. Three of these groups were fed a 5%-protein diet (starch 75·5%, casein 5·5%, sucrose 9·0%, vitamin mixture 2%, salt mixture 2% and sesame oil 6%) for 5 wk, while the other seven received stock ration. One group on the low-protein diet and one on the stock diet were then killed for bone-marrow metaphase analysis. The remaining groups were fed for a further period of 6 wk on control (non-irradiated) or test (irradiated) diets containing the various proportions of wheat shown in Table 1. The wheat was again stored for 2 wk between irradiation and use.

Experiment 3. A group of six 20-wk-old rats consumed for 7 days stock ration containing 70% irradiated wheat, which was fed to the animals within 24 hr of irradiation. Group I of experiment 2, which was maintained on stock ration containing 70% non-irradiated wheat, served as the control.

Irradiation

Wheat was irradiated at 75 krad using a cobalt-60 γ-ray source at a dose rate of 3·0 krad/min. In experiments involving the feeding of irradiated wheat within 24 hr of irradiation, the cobalt-60 γ-ray cell used delivered a dose rate of 6·3 krad/min.

Bone-marrow analyses

On completion of the feeding schedules, the animals were killed for cytological analysis. All animals were injected with colchicine (7 mg/kg body weight) 1·5–2 hr before killing to accumulate mitoses. Bone marrow tissue from the femur bones was collected in Medium 199 and was processed after treatment with hypotonic KCl and acetic–methanol fixation. The slides were prepared by an air-drying technique. Four slides were prepared from each animal, and

Table 1. *Diets fed for 6 wk to groups of rats fed for the previous 5 wk either on stock diet or on a low-protein diet (experiment 2)*

Dietary component	Level (%) in diet fed during wk 6–11 to group*						
	II	III	IV	V	VI	IX	X
Wheat flour	100†	90	90†	75	75†	90	90†
Sugar	—	—	—	9	9	—	—
Vitamin mixture	—	2	2	2	2	2	2
Salt mixture	—	2	2	2	2	2	2
Sesame oil	—	6	6	6	6	6	6
Casein		–	–	6	6	—	—
Protein content	11–12	11–12	11–12	16	16	11–12	11–12

*Groups I–VII received stock diet (16% protein) and groups VIII–X a low-protein diet (5% protein) during wk 1–5. Group I continued on the stock diet to wk 11, and groups VII and VIII were killed at the end of wk 5. For composition of stock and low-protein diet see text (Experimental).
†Wheat irradiated at 75 krad 2 wk before use.

were coded and stained in lacto-aceto-orcein. The slides were scored by two independent observers.

Statistical evaluation of the data was carried out using a *t* test and the Kolmogorov–Smirnov test of non-parametric analysis (Massey, 1951).

RESULTS AND DISCUSSION

The quality of the preparations varied from sample to sample. In most cases about 40% of the metaphases showed good chromosome spreads, about 30% were medium spreading and the rest were non-spreading types. The mitotic index ranged from 2·5 to 3·4% in these samples. The number of polyploid cells, either well-spreading or non-spreading and having 4n = 84, varied from slide to slide. In most of the polyploid cells the chromosomal complements were in a condensed state. Since the frequency of polyploid cells was low, all the metaphases, irrespective of whether they were well spread or medium spread, were included for the purpose of computing the frequency. This represented about 65–70% of all dividing cells in each slide. In the majority of samples studied more than 3000 metaphases could be scored. Some sort of clustering was evident in the distribution of polyploid cells. The percentage of polyploid cells was computed for each animal and the significance of the data obtained for the various groups was assessed.

It was evident from the results of the first experiment that the feeding of irradiated wheat at a level of 70% in the stock diet did not provoke any significant increase ($P > 0{\cdot}05$; n = 6) in the frequency of polyploid cells, the mean values obtained for the control and test groups being 0·213 ± 0·051 and 0·249 ± 0·041% respectively. A marginal decrease was observed in the ploidy levels of rats fed a low-protein diet (group VIII) compared with those on the stock diet with a protein content of 16% (group VII), but the difference was not statistically significant (Table 2). No significant increase in the frequency of polyploid cells was observed in any of the groups (II, IV, VI and X) fed diets containing different proportions of irradiated wheat (Table 2). The distribution of polyploid cells in the control and test groups was shown to be normal (Fig. 1a) by the Kolmogorov–Smirnov test. The pooled data did not suggest any bimodal distribution (Fig. 1b). In all these cases, $P > 0{\cdot}2$. A slight increase in the polyploid cells was observed in animals fed the 90% irradiated-wheat diet (group IV) but the value was neither statistically significant ($P > 0{\cdot}05$) nor attributable to the feeding of irradiated wheat, since no such increase was observed in animals fed diets containing 100 or 75% irradiated wheat. Furthermore, the feeding of freshly irradiated wheat within 24 hr of irradiation to another group of rats showed a mean percentage of 0·283 ± 0·029 polyploid cells, a value again not significantly different from the values for the relevant control group (0·217 ± 0·017; $P > 0{\cdot}05$, n = 6).

The significance of tissue-specific somatic ploidy, characteristic of highly differentiating tissues in mammals and man, is not properly understood. Polyploid cells have been found to occur in various tissues, such as liver, bone marrow and the epithelium of the urinary bladder in rats, mice, hamsters and rabbits

Table 2. *Frequency of polyploid cells in the bone marrow of rats fed diets containing different proportions of non-irradiated or irradiated wheat (experiment 2)*

Diet fed in wk 1–5	Group	Wheat flour (%) in diet fed in wk 6–11	Mean frequency of polyploid cells (%)
Stock	I	70*	0·217 ± 0·017
	II	100†	0·231 ± 0·027
	III	90	0·202 ± 0·029
	IV	90†	0·305 ± 0·037
	V	75	0·194 ± 0·032
	VI	75†	0·220 ± 0·053
	VII	—‡	0·274 ± 0·048
Low-protein	VIII	—‡	0·178 ± 0·027
	IX	90	0·227 ± 0·043
	X	90 †	0·216 ± 0·025

*Stock diet.
†Wheat irradiated at 75 krad 2 wk before use.
‡Killed at end of wk 5.
Values are means ± SEM for groups of six rats; there were no significant differences ($P > 0{\cdot}05$) between any of the groups.

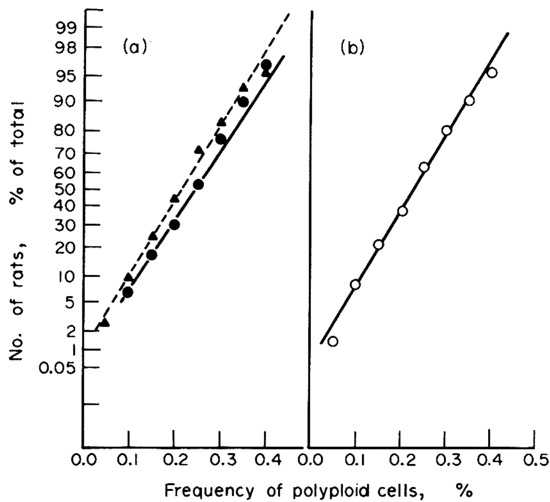

Fig. 1. Distribution of polyploid cells (4n = 84) in rat bone marrow: (a) observed and theoretical distribution of polyploid cells in control (▲---▲) and test (●——●) groups (control group, $\mu = 0{\cdot}214$, $\sigma = 0{\cdot}0923$, $D_{max} = 6{\cdot}9\%$, $P > 0{\cdot}2$; test group, $\mu = 0{\cdot}244$, $\sigma = 0{\cdot}100$, $D_{max} = 5{\cdot}6\%$, $P > 0{\cdot}2$; difference between distributions in control and test groups, $D_{max} = 18{\cdot}1\%$, $P > 0{\cdot}2$); (b) pooled data (○——○; $\mu = 0{\cdot}227$, $\sigma = 0{\cdot}091$, $D_{max} = 2{\cdot}8\%$ $P > 0{\cdot}2$).

(Alfert & Geschwind, 1958; Carriere, 1969; Ingalls & Yamamoto, 1972; Naora & Naora, 1964), but a change in their frequencies has never been considered in the toxicological evaluation of any environmental factor. The reported increase in polyploid cells in the bone-marrow of rats fed unstored irradiated wheat (Vijayalaxmi & Sadasivan, 1975) has not been confirmed during the present study. The statistical significance reported by these workers arose because of very low values seen in their control animals (0·00 and 0·10% in well fed and underfed rats, respectively); in their subsequent studies the frequency of polyploid cells in animals fed non-irradiated diet (20% protein) ranged from a mean value of 0·08 to one of 0·25% (National Institute of Nutrition, 1975). The same group of workers reported no increase in the polyploid cells of rats fed stored irradiated wheat (Vijayalaxmi, 1975). Radiation disinfestation is primarily intended for wheat that is to be stored for a considerable length of time.

In the study now reported, wheat irradiated at 75 krad and fed in different proportions in the diet induced no increase in the frequency of polyploid cells in the bone marrow of Wistar rats. No effects were observed, even after the feeding of the irradiated wheat within 24 hr of irradiation.

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Note added in proof:

In a recent paper, Frohberg & Schulze Schencking (1975) have shown that the incidence of polyploid cells differs from species to species, values of 0·16, 0·31 and 0·19% having been observed in control mice, Chinese hamsters and rats, respectively.

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AFLATOXIN PRODUCTION ON IRRADIATED FOODS

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Abstract—Irradiation has been suggested as a means of increasing the storage life of agricultural commodities but is known also to increase the susceptibility of the products to certain storage fungi. Therefore the effect of irradiation on the ability of some commonly used foods to support aflatoxin production was studied under laboratory conditions. The levels of aflatoxin were found to be significantly higher in some irradiated cereals, millets and root vegetables than in non-irradiated samples of the same food.

INTRODUCTION

Storage of food grains under unfavourable conditions is known to promote the development of storage fungi. Important among these are the Aspergillus species, mainly *A. flavus* and *A. parasiticus*. During their development, these fungi produce toxic metabolites collectively known as aflatoxins, which have hepatotoxic and carcinogenic potential. Their toxicity has been demonstrated in a variety of experimental animals (Butler, 1974), including the subhuman primate (Gopalan, Tulpule & Krishnamurthi, 1972; Tilak, 1975; Tulpule, Madhavan & Gopalan, 1964). Recently, in parts of Western India, an epidemic of hepatitis in man resulted from consumption of aflatoxin-contaminated maize (Krishnamachari, Ramesh Bhat, Nagarajan & Tilak, 1975a,b).

Many workers (Bellamy, 1959; Hannesson, 1972; Niven, 1958) have studied the use of γ-irradiation to extend the storage life of certain foods by reducing microbial populations and controlling insect and parasitic infestation. The prevention of sprouting by radiation treatment has been suggested as a means of maintaining the quality of root crops during storage (Sommer & Fortlage, 1966). Attention has frequently been focussed on possible increases in susceptibility to infection by fungi and decay following irradiation. Susceptibility could be increased as a result of a reduction in the physiological and biochemical resistance of the host tissue. Further, some fungi that are weakly pathogenic might colonize vigorously on tissues of lowered resistance (Sommer & Fortlage, 1966).

It has been suggested on the basis of recent findings that wheat subjected to irradiation needs subsequently to be stored for periods extending beyond 3 months to render it wholesome and safe for human consumption (Vijayalaxmi, 1975). During such storage of irradiated foods under conditions favouring infection with fungi such as *A. flavus*, toxin elaboration could be altered. It was considered important, therefore, to determine the effect of irradiation on aflatoxin production, and the results of a study of toxin production under laboratory conditions in irradiated foods infected with the fungus are reported here.

EXPERIMENTAL

The foods studied included wheat (*Triticum aestivum*), maize (*Zea mays*), sorghum (*Sorghum vulgare*), pearl millet (*Pennisetum typhoideum*), potatoes (*Solanum tuberosum*) and onions (*Allium cepa*). These foods were irradiated with a ^{60}Co irradiation source. The doses of irradiation used were 10 krad for potatoes and onions and 75 krad for wheat, maize, sorghum and pearl millet. These are the doses recommended for irradiation of different foods (Joint FAO/IAEA/WHO Expert Committee, 1970). The dose delivered was checked by ferrous sulphate dosimetry.

Irradiated and non-irradiated wheat, maize, sorghum and pearl millet in 10 g amounts were sterilized with 5 ml water at 121°C for 15 min. Irradiated and non-irradiated unpeeled potatoes and onions sliced into two, in 50 g lots, were also sterilized at 121°C for 15 min but without any water because of their high moisture content. *A. parasiticus* (NRRL 2999) spores were subcultured on to PDA slants. A loopful of spores from a 4-day-old culture was suspended in 20 ml sterile water. From this spore suspension (approximately 8×10^5 spores/ml), 1 ml aliquots were inoculated into each of the flasks containing the sterile irradiated or non-irradiated foods. Each flask was incubated at 27°C for 7 days, at the end of which the contents were sprayed with alcohol. The infected samples were dried overnight at 80°C, powdered, defatted with *n*-hexane and extracted with methanol. The methanol extract was depigmented by treatment with 20% basic lead acetate solution, and the clear filtered solution was extracted repeatedly with chloroform. The chloroform extracts were evaporated and made up to a known volume for quantitative estimation of aflatoxin B_1. Suitable aliquots were spotted on activated silica gel C thin-layer chromatography plates, which were developed in chloroform–methanol (98:2, v/v) and scanned for quantitation of the toxin (Pons, 1968) on a Photovolt Densitometer, 110 V (Photovolt Corp., New York).

RESULTS AND DISCUSSION

The results of the study are presented in Table 1. Following irradiation, aflatoxin production was found

Table 1. *Aflatoxin production on irradiated and non-irradiated foods by* A. parasiticus

Food	Aflatoxin B_1 ($\mu g/g$)*		
	In non-irradiated samples	In irradiated samples	Increase in irradiated foods (%)
Wheat	208·1 (196·0–213·6)	303·2 (294·3–322·0)	45·7
Maize	125·6 (120·0–131·2)	165·0 (161·0–168·3)	31·4
Sorghum	25·1 (20·1–31·0)	45·3 (42·2–48·9)	80·8
Pearl millet	27·3 (20·5–34·3)	45·3 (34·2–56·2)	66·0
Potatoes	0·85 (0·63–1·20)	1·48 (1·30–1·62)	74·4
Onions	0·70 (0·64–0·75)	1·28 (1·19–1·57)	84·0

*Values are means of three series, each series being determined in duplicate or triplicate, with figures in parenthesis indicating the range. The F ratio test of variance was done with a significance of $P < 0·01$.

to increase by 45·7% in wheat, by 31·4% in maize, by 80·8% in sorghum, by 66% in pearl millet, by 74·4% in potatoes and by 84% in onions. Thus, in all the agricultural commodities investigated, irradiated foods consistently showed an appreciable increase in aflatoxin production.

This observation must be considered of great importance to public health. Increased aflatoxin production following irradiation (100 krad) of the fungal spores of *A. flavus* has already been reported (Applegate & Chipley, 1973 & 1974a,b; Bullerman, Barnhart & Hartung, 1973; Jemmali & Giulbot, 1969 & 1970a,b) and may perhaps be explained on the basis of mutations resulting from exposure of the spores to radiation. However, the reason for the increased production of toxin by fungus grown on irradiated foods is not clear. Two possibilities may be considered. Irradiation is known to produce certain biochemical changes in the food and these may result in an increased somatic growth of the fungus and hence in an increase in aflatoxin production. These biochemical changes could also increase the availability of precursors for toxin production without necessarily affecting the somatic growth of the fungus. The observation that irradiation of wheat leads to a 12% decrease in triglyceride content with a concomitant increase in the monoglyceride level (Department of Atomic Energy, Bombay, 1974–75) and the suggestion that wheat has a saponifiable lipid fraction composed essentially of unsaturated fatty acids responsible for supporting increased production of aflatoxin (Jemmali & Guilbot, 1974) acquires significance in this context. So does the observation from the Philippines that, following irradiation, the free fatty acid levels of shelled peanuts change significantly (Food Irradiation Information, 1975).

In view of these findings, the possibility that an increased or altered fatty acid profile in irradiated foods may be responsible for increased aflatoxin production is now being investigated.

Irrespective of the mechanism, the finding that irradiated foods can support a greater production of the toxin must be viewed with concern from the public health point of view. It may be argued, however, that these studies have been carried out on irradiated foods under laboratory conditions and that the findings may not have much practical relevance. The fact is that aflatoxins have been reported to be present in samples of peanuts, maize, sorghum, rice and wheat under natural conditions as well as in potato products (Detroy, Lillehoj & Ciegler, 1971; Diener & Davis, 1969; Shank, Wogan, Gibson & Nondasuta, 1972). Accordingly, trials of irradiated foods, on a larger scale than we have attempted, should be carried out to ascertain their susceptibility to mycotoxin production.

If irradiated foods become infected with *A. flavus* during storage, as is possible, particularly if conditions of storage are not satisfactory and the moisture content increases, the risk of greater amounts of toxin being formed must be considered as being very real. This risk must be taken into consideration before irradiation is recommended as a method for improving the storage life of foods.

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TENEUR MINIMALE DU REGIME EN LINDANE INDUISANT LES MONOXYGENASES MICROSOMALES CHEZ LE RAT

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Résumé—Nous incorporons dans la nourriture de jeunes rats mâles 0,2, 0,5, 2, 20 ou 120 ppm de lindane. Le traitement dure 4 semaines. Nous prélevons et pesons le foie et les reins. Nous mesurons dans une préparation de microsomes hépatiques la *N*-déméthylation de l'aminopyrine (aminophénazone), l'hydroxylation de l'aniline, les teneurs en cytochromes *P*-450 et b_5. Le lindane augmente le poids du foie et surtout des reins; pour ces derniers la dose efficace de pesticide est inférieure à 20 ppm. Le lindane induit les monoxygénases microsomales du foie ainsi que la synthèse nette du cytochrome *P*-450 pour une teneur dans le régime comprise entre 2 et 20 ppm, inférieure donc au *no-effect level* (25–50 ppm). Cependant, l'induction enzymatique par l'insecticide ne présente pas les mêmes caractéristiques suivant le type de substrat. L'accélération du métabolisme de l'aniline (type II) résulterait essentiellement de l'augmentation de la quantité *P*-450 alors que la vitesse du métabolisme de l'aminopyrine (type I) serait accrue en partie seulement par l'excès de ce cytochrome.

Abstract—Young male rats were fed for 4 wk on a diet containing 0·2, 0·5, 2·0, 20 or 120 ppm lindane, after which livers and kidneys were removed and weighed. Aminopyrine *N*-demethylation, aniline hydroxylation and levels of cytochrome *P*-450 and b_5 were measured in a preparation of hepatic microsomes. Lindane increased the liver weight and particularly the weight of the kidneys; in the latter case the effective dose of the pesticide was below 20 ppm. Lindane induced microsomal mono-oxygenases of the liver as well as the synthesis of cytochrome *P*-450 at dietary levels of 2–20 ppm, levels below the previously established no-effect level of 25–50 ppm. However, enzyme induction by the pesticide presents different characteristics according to the type of substrate. Acceleration in aniline metabolism (type II substrate) results essentially from the increase in the quantity of *P*-450, although the rate of metabolism of aminopyrine (type I) is increased only in part by the excess of this cytochrome.

INTRODUCTION

Le lindane (isomère gamma de l'HCH) se distingue des autres insecticides organochlorés par sa solubilité relativement bonne dans l'eau, par son élimination assez rapide de l'organisme des vertébrés et surtout par sa toxicité chronique considérablement plus faible (Herbst et Bodenstein, 1972). Ses propriétés et son efficacité comme pesticide font qu'il remplace de plus en plus les autres hydrocarbures chlorés. On peut donc s'attendre à trouver une augmentation des résidus du lindane dans les prochaines années.

Dans un article précédent (Pélissier, Manchon, Atteba et Albrecht, 1975), nous avions montré qu'une alimentation contaminée par 120 ou 240 ppm de lindane entraîne, après 4 ou 8 semaines, une induction de plusieurs enzymes des microsomes hépatiques chez le rat. Nous avions remarqué en particulier une accélération des métabolismes de l'aminopyrine (aminophénazone) et de l'aniline. La DL_{50} *per os* chez le rat est de 125 mg/kg (Bailly et Dubois, 1974), les quantités de lindane que nous avions utilisées correspondent donc à une administration quotidienne d'environ 5 et 10% de cette DL_{50}.

On considère que la dose sans effet nocif à long terme (*no-effect level*) dans l'alimentation du rat est comprise entre 25 et 50 ppm (Herbst et Bodenstein, 1972). Or, d'après un travail de Kolmodin-Hedman, Alexanderson et Sjöqvist (1971), l'induction des enzymes microsomales par le lindane serait sensible, après 1 ou 2 semaines de traitement, pour une teneur dans le régime considérablement plus faible. Ces auteurs constatent une diminution du temps de sommeil produit par de l'hexobarbital chez des rattes nourries avec une régime contenant seulement 0,5 ppm de lindane. On admet que la durée de sommeil par les barbituriques dépend directement de l'activité des enzymes liées au cytochrome *P*-450 dans les microsomes hépatiques (Fouts, 1971).

Il nous a donc paru nécessaire de déterminer la quantité minimum de lindane dans le régime faisant apparaître *in vitro* une modification de l'activité des enzymes microsomales à moyen terme. Nous étudions le métabolisme de l'aminopyrine (substrat de type I) et de l'aniline (type II) chez des rats dont la nourriture est contaminée par 0,2–120 ppm de lindane. Pour apporter quelques précisions sur le mécanisme de l'induction par l'insecticide, nous mesurons les teneurs en cytochromes *P*-450 et b_5.

METHODES EXPERIMENTALES

Animaux et traitement. Notre animalerie, éclairée de 8 à 20 heures, est ventilée en continu et maintenue à une température de 22 ± 1°C. Les rats sont placés par deux dans des cages de plastique d'environ 14 dm^3. De la sciure granulaire de résineux constitue leur litière; elle est renouvelée deux fois par semaine.

Etant donné que l'on observe des variations de l'activité des enzymes microsomales suivant les saisons (Beuthin et Bousquet, 1970), nous précisons que l'expérience se déroule aux mois d'octobre et novembre.

Nous recevons toutes les 2 semaines un lot de 14 rats mâles de souche Wistar CF provenant d'un élevage à flore contrôlée *SPF*. Ce sont des animaux au sevrage à 18–20 jours. Pendant une semaine, nous leur donnons une provende commerciale (biscuits "Extralabo") qui titre 25,6% de protides; puis durant 2 semaines, les rats sont nourris avec un régime semi-synthétique équilibré, que nous préparons au laboratoire, contenant 18,2% de protides (caséine). Nous indiquons la composition complète de ce régime ainsi que son mode de préparation dans un article antérieur (Albrecht et Manchon, 1973). Toutes les matières employées pour la fabrication de ce régime sont des produits destinés à l'alimentation humaine; nous supposons donc que la teneur en lindane est inférieure à 0,05 ppm. Les 3 semaines d'acclimatation à notre animalerie étant terminée, nous répartissons au hasard les 14 rats dans sept cages et nous les pesons.

Ils reçoivent ensuite pendant 4 semaines le régime précédent additionné de 0 (pour quatre animaux témoins), 0,2, 0,5, 2, 20 ou 120 ppm de lindane pur (pour deux animaux par dose). L'insecticide est très soigneusement mélangé au régime en poudre mais n'est pas préalablement dissous dans un solvant organique.

Ce protocole expérimental est répété quatre fois. Nous disposons donc au total de 56 rats soit 16 témoins et huit traités pour chacune des doses de lindane. Le poids moyen des animaux au commencement du traitement est 125,9 ± 1,79 g; nous vérifions par une analyse de variance (Schwartz, 1966) l'homogénéité des groupes expérimentaux à ce moment: le ${}^{6}_{43}F$ de la variance intergroupes est égal à 1, 2; la disparité entre les groupes expérimentaux n'est pas significative.

A la fin du traitement, après un jeûne d'une nuit, nous pesons puis nous sacrifions les rats par décapitation, entre 9 et 11 heures, suivant un ordre déterminé par un tirage au sort (Radzialowski et Bousquet (1968) observent une variation circadienne de la *N*-déméthylation de l'aminopyrine). Le foie et les reins sont prélevés, lavés et pesés. Nous broyons le foie à l'"Ultra Turrax" pendant 30 sec dans 3 vols de saccharose 0,25 M refroidi et nous préparons des microsomes (250 mg foie/ml) selon le procédé de Kamath et Narayan (1972) tel que nous l'avons décrit (Albrecht, Pélissier, Manchon et Rospars, 1973). Nous appliquons la méthode de Lowry, Rosebrough, Farr et Randall (1951) pour déterminer la teneur en protéines des microsomes.

Activités enzymatiques. Nous indiquons dans un article précédent (Pélissier *et al.* 1975) les conditions de mesure de la *N*-déméthylation de l'aminopyrine et de l'hydroxylation de l'aniline. Nous déterminons les teneurs en cytochromes *P*-450 et b_5 des microsomes selon les méthodes que décrivent Omura et Sato (1964). Le milieu d'incubation comporte de l'HEPES 0,1 M (acide *N*-2-hydroxyéthylpipérazine-*N*-2-éthane sulfonique) à pH = 7,3 et des microsomes correspondant à 100 mg foie/ml (environ 4 mg protéines/ml).

Analyse statistique. Nos résultats sont établis par des méthodes dérivées de l'analyse de la variance (Schwartz, 1966). L'organisation de notre expérience nous permet d'appliquer la méthode des blocs complets avec répétition et de diminuer ainsi au mieux la variance résiduelle. Les tableaux indiquent la moyenne et la valeur estimée de son écart-type chez les rats témoins ainsi que le pourcentage de variation de la moyenne des animaux traités par rapport à ces témoins. Nous calculons le poids relatif moyen du foie ou des reins et son écart-type par une analyse de covariance (Lazar et Lellouch, 1971). Toutes ces analyses statistiques sont effectuées sur une calculatrice électronique (Programma 602) grâce à des programmes mis au point par R. Lowy et Ph. Manchon (1972, non publiés).

RESULTATS

Caractéristiques des animaux

Le lindane, 0,2 à 120 ppm dans le régime pendant 4 semaines, ne ralentit pas la croissance des rats (Tableau 1); nous observons même une augmentation significative du gain de poids moyen au cours du traitement avec 20 ppm.

Le poids relatif du foie est significativement plus élevé chez les animaux ingérant 0,2 et 120 ppm. L'effet de la dose faible est tout à fait inattendu; il nécessite une confirmation. Le poids relatif des reins est très significativement augmenté avec 20 et 120 ppm. L'hypertrophie, respectivement 11 et 16%, est fonction de la quantité de lindane ingérée.

Activités enzymatiques

Quatre semaines de régime contaminé par 0,2–120 ppm d'insecticide ne paraissent pas modifier la concentration des protéines microsomales du foie (Tableau 2).

Le traitement avec 120 ppm de lindane accélère sensiblement la *N*-déméthylation de l'aminopyrine

Tableau 1. *Caractéristiques des animaux*

Caractéristique	Rats témoins†	Lindane (ppm de régime)...	Rats traités au lindane (% de variation par rapport aux témoins) 0,2	0,5	2	20	120
Poids corporel (g)	259.7 ± 5.80		+0.5	+3.2	−6.4	+5.7	+7.0
Gain de poids (g/semaine)	32.8 ± 1.14		+10.0	+11.5	−2.1	+15.4*	+12.0
Poids relatif							
du foie (g/100 g rat)	4,035 ± 0.089		+11.5*	−3.6	−4.8	−1.6	+8.8*
des reins (g/100 g rat)	0,952 ± 0.021		+6.1	+3.1	−3.3	+10.9**	+16.1**

†Nombre d'animaux, 16.
Les variations marquées avec des astérisques sont significatives: *$P < 0{,}05$; **$P < 0{,}01$.

Tableau 2. *Activités enzymatiques dans les microsomes hépatiques*

Paramètre	Rats témoins†	Lindane (ppm de régime)...	Rats traités au lindane (% de variation par rapport aux témoins)				
			0,2	0,5	2	20	120
Protéines (g/100 g foie)	4,08 ± 0,126		−2,0	+2,2	+1,0	+9,8	+2,5
N-Déméthylation de l'aminopyrine‡							
nmol/100 mg protéines/min	17,3 ± 1,45		+1,2	−12,7	+2,9	+24,9	+95,4**
nmol/nmol *P*-450 min	0,283 ± 0,022		−1,8	−11,7	+6,4	+7,4	+46,6**
Hydroxylation de l'aniline§							
nmol/100 mg protéines/min	25,0 ± 1,01		−2,0	−3,2	+2,0	+16,0*	+46,4**
nmol/nmol *P*-450/min	0,399 ± 0,018		+1,5	+2,0	+6,8	+5,3	+15,5
Cytochrome *P*-450							
nmol/100 mg protéines microsomales	62,6 ± 2,22		−0,8	−3,8	−3,2	+12,9*	+30,5**
Cytochrome b_5							
nmol/100 mg protéines microsomales	83,6 ± 1,61		−2,9	−3,8	+0,5	0	−5,9

†Nombre d'animaux, 16.
‡nmol de 4-aminoantipyrine/100 mg protéines microsomales ou nmol *P*-450/min.
§nmol de *p*-aminophénol/100 mg protéines microsomales ou nmol *P*-450/min.
Les variations marquées avec des astérisques sont significatives: $*P < 0,05$; $**P < 0,01$.

dans les microsomes (Tableau 2). Nous observons une augmentation très significative de l'activité enzymatique rapportée aux protéines ou au *P*-450 (cette dernière expression représente l'activité moléculaire spécifique ou *turnover number*). Si nous exprimons l'activité pour 100 mg de foie, nous remarquons alors une induction même avec 20 ppm: 0,966 ± 0,0946 (8) nmol de 4-aminoantipyrine formée/min, au lieu de 0,700 ± 0,0669 (16) chez les témoins; il en est de même pour l'activité totale par foie: respectivement 103,3 ± 9,87 (8) et 73,1 ± 6,98 (16) nmol.

L'hydroxylation de l'aniline est accélérée chez les rats traités avec 20 et 120 ppm de lindane lorsque l'activité enzymatique est rapportée aux protéines (Tableau 2). L'induction, respectivement de 16 et de 46%, dépend de la quantité d'insecticide ingéré. Une teneur de lindane dans le régime inférieure ou égale à 2 ppm est sans effet apparent. Contrairement au métabolisme de l'aminopyrine, l'activité moléculaire spécifique (rapportée au *P*-450) de l'hydroxylation de l'aniline ne paraît pas sensiblement modifiée, même avec 120 ppm.

La quantité de *P*-450 des microsomes est significativement augmentée avec 20 ppm de lindane; elle l'est très significativement avec 120 ppm; l'induction est plus importante avec la dose forte (Tableau 2). Nous constatons qu'une teneur d'insecticide égale ou inférieure à 2 ppm ne modifie pas la concentration du *P*-450 dans les microsomes.

Le lindane ne semble pas influencer sensiblement la biosynthèse nette du cytochrome b_5 dans nos conditions expérimentales.

DISCUSSION

Effets sur la croissance et sur le poids des organes

Dans nos conditions expérimentales, des rats mâles reçoivent 0,2 à 120 ppm de lindane dans leur régime pendant 4 semaines. D'après le travail de Fitzhugh, Nelson et Frawley (1950), ces quantités d'insecticide ne sont pas suffisantes pour diminuer la consommation alimentaire. D'autre part, avec une telle durée de traitement, il devrait s'établir un équilibre entre l'accumulation de lindane dans les organes et son élimination (Sieper, 1972).

Nous ne remarquons pas de ralentissement de la croissance corporelle. Dans notre expérience précédente (Pélissier *et al.* 1975), nous notions une légère baisse avec 240 ppm alors que Fitzhugh *et al.* (1950) signalaient qu'il faut plus de 800 ppm pour observer un tel effet après 6 mois d'administration.

Le lindane tend à augmenter le poids relatif du foie chez le rat. L'hypertrophie et/ou l'hyperplasie serait sensible pour une teneur comprise entre 20 et 120 ppm. Remarquons que cet effet dépend de l'espèce animale puisque Wagstaff et Street (1971) observaient, au contraire, une diminution du poids relatif du foie chez le Cochon d'Inde traité par 25 ppm d'insecticide pendant 2 semaines.

Un fait, qui à notre connaissance n'est pas signalé dans la littérature, est l'augmentation du poids des reins par le lindane. Dans cette expérience, nous constatons un excès de poids de 11% avec 20 ppm et de 16% avec 120 ppm. Nous avions déjà noté (Pélissier *et al.* 1975) des augmentations de 17 et 32% avec respectivement 120 et 240 ppm. L'effet de l'insecticide sur le poids des reins se manifeste à partir d'une valeur comprise entre 2 et 20 ppm: inférieure donc au *no-effect level* (25–50 ppm) établi par Herbst et Bodenstein (1972). Nous ne savons pas quel est, dans ce cas, le sens physiopathologique de ce phénomène. Faut-il rapprocher de l'hyperplasie qu'observait Constantinides (1951) lors d'un stress aigu?

Nous avons par ailleurs signalé qu'une quantité de lindane dans le régime égale ou inférieure à 240 ppm ne paraît pas modifier le poids du coeur, de la rate, des testicules, du cerveau ou du thymus après 2, 4 ou 8 semaines d'administration chez le rat (Ph. Manchon, R. Albrecht et M. A. Pélissier, Rapport (non-publié) au Comité "Contamination des Chaînes Biologiques", Ministère de l'environnement, Paris, 1974; Pélissier *et al.* 1975).

Effets sur l'activité des enzymes microsomales liées au P-450

Récemment den Tonkelaar et van Esch (1974) ont donné pendant 2 semaines à des rats mâles des régimes contaminés par 2–200 ppm de divers pesticides, dont le lindane. Ils ont mesuré la *N*-déméthylation de l'aminopyrine, l'hydroxylation de l'aniline et l'oxydation de l'hexobarbital dans un surnageant postmitochondrial (10 000 g) de foie. Les résultats que nous présentons dans cet article (obtenus à partir de préparations de microsomes) diffèrent peu dans leur

ensemble de ceux de ces auteurs. Cependant, le fait essentiel est que nous observons une accélération du métabolisme microsomal de l'aniline pour une quantité de lindane plus faible. Selon nous, l'hydroxylation de l'aniline est accélérée chez les rats traités avec 20 et 120 ppm. La dose minimum d'insecticide ayant un tel effet est donc comprise entre 2 et 20 ppm. Den Tonkelaar et van Esch (1974) notaient une induction avec 200 ppm mais pas avec 50 ou 20 ppm. D'autre part, nous observons une accélération de la *N*-déméthylation de l'aminopyrine chez les rats traités avec 120 ppm de lindane et peut-être même avec 20 ppm. Den Tonkelaar et van Esch (1974) constataient une induction avec 50 ppm mais pas avec 20.

L'aminopyrine et l'aniline sont des substrats des enzymes microsomales respectivement de type I et de type II. On sait que leur métabolisme se réalise par des mécanismes qui diffèrent (Mannering, 1971). Il nous paraît intéressant de noter que les inductions par le lindane ne présentent pas les mêmes caractères suivant le type de substrat. En effet, l'activité moléculaire spécifique, rapportée au *P*-450, lors de la *N*-déméthylation de l'aminopyrine est plus élevée que celle des témoins (tout au moins avec 120 ppm) alors que l'activité moléculaire spécifique pour l'hydroxylation de l'aniline n'est pas sensiblement modifiée par le traitement. En d'autres termes, l'accélération du métabolisme de l'aniline résulterait essentiellement de l'augmentation de la quantité de *P*-450 tandis que la vitesse du métabolisme de l'aminopyrine serait accrue en partie seulement par l'excès de ce cytochrome. La biotransformation de l'aminopyrine doit dépendre largement de la vitesse du transfert des électrons du NADPH vers le *P*-450; le lindane accélère d'ailleurs ce transfert en induisant la NADPH–*P*-450 (cytochrome *c*) réductase (Pélissier *et al.* 1975). Pour une compréhension fine des perturbations produites par l'insecticide sur le système microsomal de transport d'électrons, notre recherche est incomplète; il nous faudrait connaître aussi les modifications de la NADPH oxydase, de la NADH-b_5 réductase, de la liaison des substrats au *P*-450, de la teneur en phosphatidylcholine; nous orientons nos études actuelles dans ce sens.

Kolmodin-Hedman *et al.* (1971) ont observé une diminution du temps de sommeil produit par l'hexobarbital chez la ratte Sprague–Dawley nourrie avec un régime contaminé avec 0.5 ppm de lindane pendant 1 ou 2 semaines. Den Tonkelaar et van Esch (1974) et nous-même ne retrouvons pas une influence sur les enzymes microsomales pour une quantité aussi faible de lindane. A notre connaissance, il s'agit d'ailleurs là de la plus petite valeur d'insecticide organochloré dans l'alimentation (0,5 ppm) proposée comme ayant un effet; ce qui reviendrait à proposer que le lindane est un inducteur plus puissant que le chlordane, le DDT, la diéldrine et l'aldrine. Ce n'est pas là l'opinion commune (Fouts, 1970). Kolmodin-Hedman *et al.* (1971) n'ont pas en fait mesuré *in vitro* l'activité des monoxygénases microsomales. On pense que la détermination *in vitro* de l'activité des enzymes liées au *P*-450 constitue une épreuve plus sensible pour révéler une induction; ce qui apparemment serait l'inverse ici puisque den Tonkelaar et van Esch (1974) n'observaient une accélération de l'oxydation de l'hexobarbital *in vitro* qu'à partir de 50 ppm de lindane après 2 semaines de traitement. Un facteur 100 sépare donc les résultats de Kolmodin-Hedman *et al.* (1971) de ceux de den Tonkelaar et van Esch (1974). Si l'on tient compte de nos propres observations—par exemple, l'augmentation du *P*-450 avec 20 ppm—il demeure quand même un facteur 40. Comment expliquer un tel écart? Bien entendu, on peut mettre en cause des différences dues aux conditions d'élevage, à la race des rats (Wistar et Sprague–Dawley) et surtout au sexe. Ne peut-on pas non plus considérer qu'il y a là une véritable discordance des résultats *in vivo* et *in vitro*? Nous proposons une hypothèse qui nécessite des confirmation expérimentales. Chez l'animal témoin, l'effet pharmacologique (durée de sommeil) de l'hexobarbital dépend exclusivement de la vitesse de son métabolisme dans les microsomes du foie; chez le rat traité par du lindane, il y aurait en plus un métabolisme extrahépatique. Etant donné que le traitement par l'insecticide augmente sensiblement le poids des reins, le tissu rénal ne deviendrait-il pas alors aussi un site privilégié pour le métabolisme du barbiturique?

En conclusion, la teneur minimale du régime en lindane nécessaire pour induire les monoxygénases microsomales du foie, chez le rat mâle, est comprise entre 2 et 20 ppm pour 4 semaines de traitement. Cette induction enzymatique s'accompagne d'une hypertrophie des reins.

Ces phénomènes traduisent-ils une adaptation de l'organisme et disparaissent-ils sans dommage pour l'animal lorsqu'on maintient son exposition au pesticide? On sait que les études permettant d'établir que le *no-effect level* du lindane est de 25 ppm ont été faites sur une longue période, 2 ans ou plus, chez le rat (Herbst et Bodenstein, 1972). Cela a permis de proposer la dose journalière admissible (DJA) pour l'homme: 0.0125 mg/kg de poids corporel. Il est clair que le *no-effect level* s'obtient avec des rats ayant un régime bien équilibré et vivant dans une animalerie convenablement éclairée et climatisée. Si de nouvelles recherches permettaient de définir des circonstances physiopathologiques ou de l'environnement pour lesquelles le risque apporté par le pesticide est plus ou moins grand, il deviendrait plus aisé de dégager la signification de nos résultats.

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PHOTOTOXICITY AND PHOTOCARCINOGENESIS: COMPARATIVE EFFECTS OF ANTHRACENE AND 8-METHOXYPSORALEN IN THE SKIN OF MICE

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Abstract—Erythema was produced in the skin of hairless mutant mice by a single exposure to a solar simulator ($\lambda > 290$ nm). Topical pretreatment of the mice with either anthracene or 8-methoxypsoralen (8-MOP) intensified the reaction more than did pretreatment with the vehicle (methanol) alone. Skin tumours were produced in hairless mice during several months of daily topical treatment followed by exposure to the solar stimulator. Compared with the vehicle-treated group, the 8-MOP group developed more tumours and had a shorter tumour latent period. Tumour production in the anthracene group was not significantly different from that of the vehicle-treated group. Under the conditions of this test, enhanced photocarcinogenesis is not a necessary consequence of prolonged contact with the phototoxic agent anthracene.

INTRODUCTION

Several chemical agents have been shown to intensify the sensitivity of skin to visible or to ultraviolet radiation (UVR). A few of these photosensitizers induce an immune reaction (photoallergy); the rest provoke a response (phototoxicity) which is the photobiological analogue of primary irritation.

Cutaneous phototoxic agents also exhibit other types of photodynamic activity such as lethality, haemolysis, enzyme deactivation and protein denaturation, which have been reviewed by Santamaria & Giordano (1969) and Santamaria & Prino (1964). The association of photodynamic activity with blastogenic activity in several compounds (Doniach & Mottram, 1937; Mottram & Doniach, 1938) led to studies of the possible association of photodynamic and carcinogenic activity (Clark, 1964; Doniach & Mottram, 1940; Morton, Luce-Clausen & Mohoney 1942; and many others). However, Epstein, Small, Falk & Mantel (1964) showed that even within the polycyclic class of hydrocarbons the correlation of photodynamic and carcinogenic activity was far from perfect. Previously, Cook & Martin (1940) had shown that the ability of polycyclic aromatics to undergo photo-oxygenation was apparently unrelated to carcinogenic activity. Attempts to evaluate the interaction of photoactive carcinogenic agents and light (both carcinogenic and non-carcinogenic) have led to a large and contradictory body of literature (for reviews see Blum, 1964, Epstein, 1970, and Santamaria & Giordano, 1969; for literature prior to 1940 see Doniach & Mottram, 1940).

A less complex but equally significant problem concerns photo-induced carcinogenesis following application of agents that are phototoxic but are not themselves carcinogenic. Büngeler (1937a,b) reported that eosin and haematoporphyrin, as well as tar emulsion, could enhance the ability of sunlight to produce cutaneous tumours in mice. Heller (1950) reported that mice treated topically with anthracene followed by UVR showed necrotizing phototoxic skin responses followed by neoplasia. He concluded, in fact, that photodynamic reaction should be recognized as a new carcinogenic principle ("Durch Bestrahlung mit einer nicht krebserzeugenden Strahlung nach Sensibilisierung mit einem nicht kanzerogenen Kohlenwasserstoff konnten erstmalig in eindeutiger Versuchsanordnung bösartige Geschwülste mit Sicherheit hervorgerufen werden. Damit muss die photodynamische Lichtreaktion als neues krebserzeugendes Prinzip anerkannt werden"; Heller, 1950). However, Miescher (1942) did not succeed in producing tumours in mice exposed on 5 days/wk to a phototoxic combination of UVR and anthracene; he concluded that there was no basis for viewing photodynamic action as a new specific cancer principle ("Es besteht jedenfalls vorerst kein Grund, in der photodynamischen Lichtreaktion ein neues spezifisches Krebsprinzip zu erblicken"). These opposing conclusions are not readily reconciled.

The introduction of 8-methoxypsoralen (8-MOP) as a therapeutic agent for certain human skin diseases was followed by reports that it could enhance photocarcinogenesis in mice (Griffin, Hakim & Knox, 1958; Urbach, 1959). These demonstrations had the experimental advantage of newer and more convenient lamps which produced less stress on the animals, limited skin ulceration and improved animal survival. Evidence indicated that carcinogenesis could result from the interacting effects of a compound and irradiation with a particular waveband of light, neither of these agents being a primary carcinogen (Griffin *et al.* 1958; Hakim, Griffin & Knox, 1960).

A portion of the sunlight spectrum is carcinogenic even in the absence of an exogenous photosensitizer. At the current rate of introduction of new compounds into the environment, it has become increasingly important to determine whether a readily demonstrable property such as phototoxicity can be used to predict

compounds or treatment régimes that could enhance photocarcinogenesis. This report is based on the examination of two widely recognized phototoxic agents, 8-MOP and anthracene. These were selected because they were available in 99% pure form, leaving no doubt as to the identity of the active ingredient; they represented different classes of chemicals inducing similar but not identical physiological responses (Allison, Magnus & Young, 1966) and neither was a chemical carcinogen.

The results of the study are pertinent to the opposing conclusions of Miescher (1942) and Heller (1950). The development of more appropriate light sources (solar simulators), animals (hairless mice) and test procedures (subacute phototoxicity) has, in fact, made possible a type of study envisaged by Miescher (1942).

EXPERIMENTAL

Animals. Genetically hairless (*hr*) mice of an Skh: hairless-1 outbred stock (Forbes & Urbach, 1975) were placed in isolation cages (Forbes & Urbach, 1969) when they were 6 wk of age. They had free access to tap-water and mouse chow throughout the experiment.

Radiation source. The solar simulator contained a 6-kW long-arc xenon burner (Osram XBF 6000) with a filter to eliminate UVR of wavelengths shorter than 290 nm and to attenuate the infra-red emission (Atlas Electric Co., Chicago, Ill.). The lamp emission spectrum was determined by means of a Cary model 14 recording spectrophotometer fitted with a radiometer accessory, and using an NBS traceable quartz-halogen reference source. The emission spectrum, determined at 2 m from the lamp, is shown in Fig. 1. The UVR flux at the exposed surface of the cage was also calculated as a function of current flow from a Westinghouse (Bloomfield, N.J.) WL767 zirconium phototube measured with a Keithley picoammeter. In simulated sunlight, the phototube has response characteristics that closely parallel the action spectrum for 'minimal erythema' of untanned, white human skin. This device yields information on the erythema effective energy (EEE) of appropriate light sources (Forbes & Urbach, 1975). Measured in this way, the flux was found to be 0·042 and 0·168 W/m^2 (EEE) at 2 m and 1 m below the lamp, respectively.

Reagents and application. Anthracene (Fisher No. 480) and 8-MOP (Paul B. Elder Co., Bryan, Ohio)

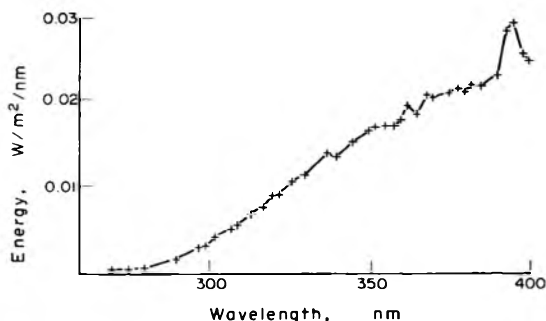

Fig. 1. Emission spectrum of xenon arc solar simulator expressed as flux at the cage surface (2 m from source).

were dissolved in reagent-grade methanol at 0·1 g/litre and stored in the dark. About 20 cm^2 of skin on the back of each mouse was treated with 40 μl of a test solution (or of vehicle alone) prior to radiation exposure.

Experimental design. For phototoxicity testing, three groups of 12 mice were pretreated once as outlined above with one of the test solutions. Animals were then immobilized 1 m below the lamp. A circular area of skin 1 cm in diameter and centered in the chemically treated area was exposed, the remainder of the animal being covered with aluminium foil. The animals received one 40-min exposure (403 J/m^2 EEE) and were examined 4, 8, 24, 48, 72 and 96 hr after exposure.

For the carcinogenesis experiment three groups of 24 mice were pretreated once daily (Mondays to Fridays) as outlined above with one of the test solutions, and were returned to their isolation cages. The cages were positioned 2 m below the light source, and exposed for 2 hr daily, Mondays to Fridays, for a maximum surface dose of 300 J/m^2 (EEE) for 38 wk. The order in which the mice were treated and their positions under the lamp were rotated daily to avoid unintentional systematic effects. The actual skin dose was influenced by the motion and position of mice during exposure; under otherwise comparable conditions, the surface dose required to produce erythema is about twice as great for free-moving mice as for immobilized mice.

Data and analysis. Mice were numbered consecutively by toe clipping and were placed randomly into isolation cages by age and sex. At the beginning of the experiment, and subsequently once weekly, each mouse was weighed and the skin was examined in detail. For each mouse, a record was prepared bearing images of the mouse viewed dorsally, ventrally and from each side. Any change in skin appearance was noted at the appropriate location on the diagram, physical features and tattoo spots being used as landmarks. A tumour could be distinguished from cysts and other surface features when the growth was approximately 0·5 mm in height or diameter. Tumours and other lesions were removed from mice that died during the experiment, all surviving animals being killed and autopsied at 38 wk. Tissues were processed for routine histological analysis.

In reports of results, "tumour prevalence" means the proportion of surviving animals bearing one or more tumours at the time of observation. In this parameter, no weighting factor is given for animals bearing more than one tumour or for the length of time that a tumour had been visible. A measure of development time for first tumours appearing on animals is available from prevalence figures. Prevalence within each group for each week was compared by the chi-square test. Median development time of first tumours (i.e. 50% tumour prevalance) was also evaluated by comparing the times of appearance of first tumours by the Wilcoxon rank sum test (Wilcoxon & Wilcox, 1964). For purposes of this comparison animals that died without developing tumours were omitted, whereas animals that survived without developing tumours were considered to have developed a tumour 1 wk after the end of the experiment. The resulting comparisons represent minimum differences.

Tumour yield is shown as the number of tumours present divided by the number of surviving mice (i.e. average number of tumours/mouse). For each observation period, the groups were compared using the Wilcoxon rank sum test (Wilcoxon & Wilcox, 1964).

Survival is expressed as the absolute number of mice alive at the time of observation, 24 being the initial number in each group. Groups were compared at each observation period by the chi-square test.

RESULTS AND DISCUSSION

Phototoxicity experiment

Without irradiation, none of the solutions provoked a visible change in skin. Areas treated with the vehicle and exposed to UVR developed barely perceptible oedema and erythema within 24 hr of exposure and the affected skin returned to normal appearance within 72 hr. Skin pretreated with 8-MOP developed a more severe response, with the greatest intensity of oedema and inflammation occurring between 48 and 72 hr after exposure and resolution of the reaction being completed between 5 and 7 days after exposure. Anthracene also provoked a more severe light response than did the vehicle: inflammatory changes were visible by 6 hr and, qualitatively, the response was a brighter red than that produced in 8-MOP treated areas. The anthracene-enhanced response was no longer visible after 48 hr. Thus, both 8-MOP and anthracene were phototoxic in this system, although their stimulated responses were not qualitatively identical.

Carcinogenesis experiment

The UVR-exposed skin pretreated with 8-MOP or with anthracene became distinguishable from the surrounding skin after about 5 wk of exposure, the treated areas having more erythema, hyperplasia, and dry desquamation than those untreated. This effect was not seen in areas treated with vehicle only. The photosensitized reaction faded gradually, and by wk 10 the three groups were again indistinguishable. However, all of these irradiated animals had skin that appeared somewhat thicker and smoother than that of unexposed mice of comparable age.

Compared with the vehicle alone, the 8-MOP solution, but not the anthracene solution, markedly enhanced photocarcinogenesis (Fig. 2). The times to 50% prevalence for the vehicle-treated and anthracene groups were 27·2 and 28·2 wk, respectively. These values were not significantly different, but the time of 20·04 wk to 50% prevalence for the 8-MOP group differed significantly from each of the other two groups ($P < 0·01$). Similarly, tumour prevalence was significantly ($P < 0·05$) greater in the 8-MOP group from 18 to 28 wk. From wk 18, tumour yield was significantly greater in the 8-MOP group than in either of the other groups ($P < 0·01$).

Most of the tumours in all groups developed near the midline of the back, very few developing on the head, snout, extremities or abdomen. Of the 50 tumours analysed histologically from each group, no fewer than 45 were squamous-cell carcinomas, the remainder including sarcomas, haemangiomas and spindle-cell tumours. The three groups did not differ in the types of tumours produced, nor was there any

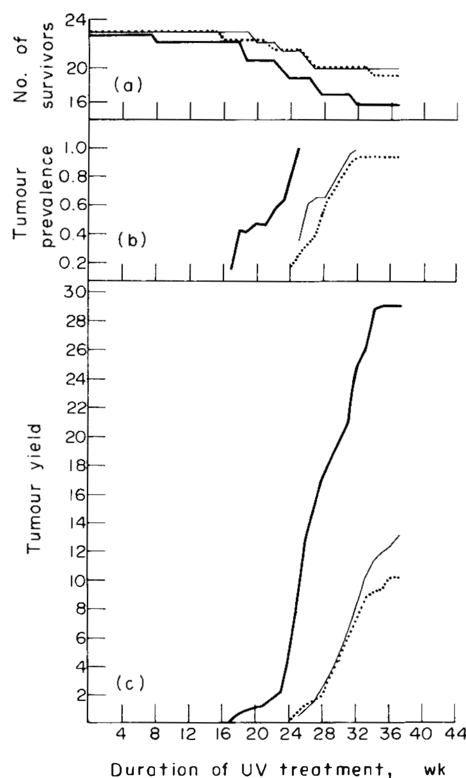

Fig. 2. Effects of pretreatment with 8-MOP (——), anthracene (······) or the vehicle (——) on (a) the survival, (b) the proportion of mice with tumours at any given time and (c) the average no. of tumours/survivor in groups of irradiated mice.

noticeable difference among groups in the tendency of tumours to enlarge and become invasive. There was no indication of distant metastasis in any group. The anthracene groups and the vehicle group did not differ significantly in terms of longevity and although there was some indication of increased mortality in the 8-MOP group, the differences were not statistically significant. Although there was little direct evidence that tumours were an immediate cause of death, the animals with large multiple tumours eventually became cachectic, and lethargy preceded death.

Under the conditions of the experiment, both test compounds were phototoxic, but only 8-MOP enhanced photocarcinogenesis. Although the compounds produced an early transient skin effect, tumours developed in the absence of severe skin damage. The epidermis, although hyperplastic, displayed orderly stratification, and inflammation was negligible. There was no ulceration of the skin during the course of the experiment.

In a number of studies on experimental photocarcinogenesis the animals experienced severe skin damage. Miescher (1942) suggested, in fact, that the chemical enhancement of photocarcinogenesis reported earlier (Büngeler, 1937a) was due to nonspecific acceleration of the UVR effect by trauma, not to a specific photodynamic action. The competing risk of treatment-related mortality (Büngeler, 1937a; Heller, 1950) also increases the difficulty of evaluating the enhancement of photocarcinogenesis by certain compounds. Heller (1950) reported no change in the

skin of mice treated with anthracene or exposed to UV-A ('long'-wave UVR, $\lambda > 320$ nm) alone, but skin cancers were produced in areas where anthracene treatment was followed by UV-A exposure. Tumours appeared very early (39–42 days after the first exposure) and were preceded by a severe acute exudative response. In fact, the acute response itself may have played a significant role in the carcinogenic process. The influence of trauma on skin carcinogenesis has not been systematically investigated, and acute phototoxicity may well be one of the several types of severe trauma that can lead to skin-tumour formation. Because severely traumatized mouse skin is particularly prone to early tumour production (Forbes, 1974; Hsu, Forbes, Harber & Lakow, 1975), the treatment schedule for this study was designed to evaluate the influence of low-level phototoxicity on long term photocarcinogenesis.

In conclusion, we have shown that 8-MOP was phototoxic to the skin of hairless mice and that chronic exposure to the compound augmented photocarcinogenesis. In contrast, another phototoxic agent (anthracene) tested in the same way did not augment photocarcinogenesis.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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ALANTROOT OIL

Synonyms: Elecampane oil; oil of Inula.
Description and physical properties: A brown liquid. The greatest part of oil of elecampane consists of a mixture of alantolactone and isoalantolactone (Guenther, 1952).
Occurrence: Found in the roots of *Inula helenium* L. (Fam. Compositae) (Gildemeister & Hoffman, 1961; Guenther, 1952).
Preparation: By steam distillation of the roots of *Inula helenium* (Guenther, 1952).
Uses: In public use before the 1900s.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·002	0·08
Maximum	0·05	0·005	0·025	0·4

Status

Elecampane is approved by the FDA for food use (21 CFR 121.1163 in alcoholic beverages only). The Council of Europe (1974) included elecampane in the list of substances, spices and seasonings deemed admissible for use, with a possible limitation of the active principle in the final product.

Biological data

Irritation. Alantroot oil tested at 4% in petrolatum produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1975).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced extremely severe allergic reactions in 23 out of 25 subjects after the second induction application (Kligman, 1975). Subjects previously sensitized to costus root oil gave severe cross-sensitization responses to alantroot oil (Epstein, 1975).

Alantolactone (one of the main constituents of alantroot) elicited positive patch-test responses in sensitized guinea-pigs (Hausen & Schulz, 1975; Schulz, Hausen, Wallhofer & Schmidt-Loffler, 1975). Two individuals initially sensitized to purified alantolactone (derived from Inula, Compositae family) showed positive patch-test reactions to costus root oil (Mitchell, 1974). Alantolactone produced positive patch-test reactions in five patients who were allergic to Frullania (Mitchell, Fritig, Singh & Towers, 1970). Hjorth (1970) sensitized four patients out of 25 with a single patch test with a 1% petrolatum dispersion of alantolactone. That a patch test with alantolactone can cause sensitization was reported also by Foussereau, Muller & Benezra (1975).

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Foussereau, J., Muller, J. C. & Benezra, C. (1975). Contact allergy to *Frullania* and *Laurus nobilis*: Cross-sensitization and chemical structure of the allergens. *Contact Dermatitis* **1**, 223.
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Guenther, E. (1952). *The Essential Oils*. Vol. V, p. 453, D. Van Nostrand, Inc., Princeton, New Jersey.
Hausen, B. M. & Schulz, K. H. (1975). Experimental studies on the identification of chrysanthemum allergies. *Contact Dermatitis* **1**, 244.

*The most recent of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology*, Special Issue II, December 1975.

Hjorth, N. (1970). Active sensitization with alantolactone. *Contact Dermatitis Newsl.* **4**, 175.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1975). Report to RIFM, 10 June.
Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.
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Mitchell, J. C., Fritig, B., Singh, B. & Towers, G. H. N. (1970). Allergic contact dermatitis from Frullania and Compositae. *J. invest. Derm.* **54**, 233.
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FENNEL OIL, BITTER

Description and physical properties: *Food Chemicals Codex* (1972). The main constituent of bitter fennel oil is anethole (Guenther, 1950).
Occurrence: Found in the seeds of *Foeniculum vulgare* Mill. var. vulgare (Mill.) Thellung (Fam. Umbelliferae) (Guenther, 1950).
Preparation: By steam distillation of the crushed seeds of *Foeniculum vulgare* Mill. var. vulgare (Mill.) Thellung (Gildemeister & Hoffman, 1961; Guenther, 1950).
Uses: In public use before the 1900s. 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	0·001	0·005	0·04
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 75-59; infra-red curve, RIFM no. 75-59.

Status

Bitter fennel oil is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 4·52 ml/kg (4·06–5·02 ml/kg) (Levenstein, 1975). The acute dermal LD_{50} value in rabbits was reported as >5 ml/kg (Levenstein, 1975).

Irritation. Fennel oil, bitter, applied undiluted to the backs of hairless mice and swine was not irritating (Urbach & Forbes, 1975), but applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, it was irritating (Levenstein, 1975). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1975).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced sensitization reactions in three of the 25 test subjects (Kligman, 1975).

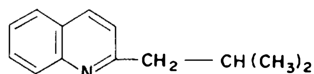
Phototoxicity. No phototoxic effects were reported for undiluted fennel oil, bitter, on hairless mice and swine (Urbach & Forbes, 1975).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19** (2), part 2, 155.
Food Chemicals Codex (1972). 2nd Ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.
Gildemeister, E. u. Hoffman, F. (1961). *Die Ätherischen Öle*. Vol. VI, p. 433. Akademie Verlag, Berlin.
Guenther, E. (1950). *The Essential Oils*. Vol. IV, p. 634. D. Van Nostrand, Inc., Princeton, New Jersey.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1975). Report to RIFM, 15 May.
Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.
Levenstein, I. (1975). Report to RIFM, 30 May.
Urbach, F. & Forbes, P. D. (1975). Report to RIFM, 18 June.

ISOBUTYL QUINOLINE

Synonyms: 2-Isobutylquinoline; α-isobutylquinoline.
Structure:



Description and physical properties: *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From acrolein and secondary butylaniline followed by dehydration and oxidation (Bedoukian, 1967).,
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·0005	0·0015	0·04
Maximum	0·05	0·005	0·02	0·2

Analytical data: Gas chromatogram, RIFM no. 72-169; infra-red curve, RIFM no. 72-169.

Status

Isobutyl quinoline is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 1·02 g/kg (0·78–1·26 g/kg) (Moreno, 1973). The acute dermal LD_{50} value in rabbits was reported as >5 g/kg (Moreno, 1973).

Irritation. Isobutyl quinoline applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1973). Tested at 2% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Kligman, 1973). Applied full strength for 48 hr in the standard occluded aluminium patch test used by the North American Contact Dermatitis Research Group, it did not produce any irritation in a 62-yr-old subject with a perfume dermatitis (Larsen, 1975).

References

Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics*. 2nd Ed., p. 320. Elsevier Publishing Co., New York.

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd Ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery*. 2nd Ed., p. 203. Givaudan-Delawanna, Inc., New York.

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

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

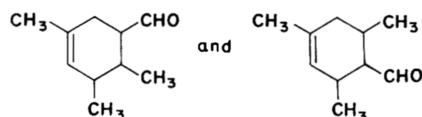
Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Larsen, W. G. (1975). Cosmetic dermatitis due to a perfume. *Contact Dermatitis* **1**, 142.

Moreno, O. M. (1973). Report to RIFM, 1 February.

ISOCYCLOCITRAL

Synonyms: 1,3,5-Trimethyl-3-cyclohexene-1-carboxaldehyde; 2,3,5-trimethyl-4-cyclohexene-1-carboxaldehyde.
Structure:



Description and physical properties: The *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By a Diels–Alder-type condensation of 2-methyl-1,3-pentadiene with crotonaldehyde (Bedoukian, 1967).
Uses: In public use since the 1950s. 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	0·08
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 72-170; infra-red curve, RIFM no. 72-170.

Status

Isocyclocitral is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported to be 4·5 ml/kg (4·16–4·86 ml/kg) (Levenstein, 1973a). The acute dermal LD_{50} value was reported to be >5 ml/kg in the rabbit (Levenstein, 1973b).

Irritation. Isocyclocitral applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Levenstein, 1973b). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics*. 2nd Ed., p. 372. Elsevier Publishing Co., New York.

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd Ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery*. 2nd Ed., p. 205. Givaudan-Delawanna, Inc., New York.

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

Kligman, A. M. (1972). Report to RIFM, 1 November.

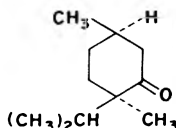
Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Levenstein, I. (1973a). Report to RIFM, 9 January.

Levenstein, I. (1973b). Report to RIFM, 16 February.

ISOMENTHONE

Structure:



Description and physical properties: A colourless slightly oily liquid.
Occurrence: *d*-Isomenthone has been reported to have been isolated from *Micromeria abissinica* Benth., *Pelargonium tomentosum* Jacquin and others. *l*-Isomenthone has been identified in *Reunion geranium*, *Pelargonium capitatum* and others (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Preparation: By hydrogenation of piperitone.
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 3000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·003	0·08
Maximum	0·2	0·02	0·05	0·8

Analytical data: Gas chromatogram, RIFM no. 72-172; infra-red curve, RIFM no. 72-172.

Status

The Council of Europe (1974) included isomenthone at a level of 0·5 ppm 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 dermal LD_{50} in rabbits was reported as >5 ml/kg (Levenstein, 1973).

Irritation. Isomenthone applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Levenstein, 1973). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

Metabolism. *d*-Isomenthone undergoes reduction in the rabbit, since *d*-isomethylglucuronide is excreted in the urine. The other possible reduction product, *d*-neoisomenthol is not excreted (Williams, 1959).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List 2, no. 2259, p. 337. Strasbourg.
Fenaroli's Handbook of Flavor Ingredients (1971). Edited by T. E. Furia and N. Bellanca. p. 493. Chemical Rubber Co., Cleveland, Ohio.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1973). Report to RIFM, 9 May.
Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis*. **1**, 231.
Levenstein, I. (1973). Report to RIFM, 16 February.
Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds*. 2nd Ed., p. 526. Chapman & Hall, Ltd., London.

2-ISOPROPYL-5-METHYL-2-HEXENE-1-AL

Synonym: Isodihydrolavandulyl aldehyde.
Structure: $CH_3 \cdot CH(CH_3) \cdot CH_2 \cdot CH{:}C(CH \cdot (CH_3)_2) \cdot CHO$.
Description and physical properties: A colourless oily liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By aldol condensation of isovaleraldehyde.
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·1
Maximum	0·15	0·015	0·04	1·0

Analytical data: Gas chromatogram, RIFM no. 72-68; infra-red curve, RIFM no. 72-68.

Status

2-Isopropyl-5-methyl-2-hexene-1-al was given GRAS status by FEMA (1973).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. 2-Isopropyl-5-methyl-2-hexene-1-al applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1973). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Flavoring Extract Manufacturers' Association (1973). Survey of flavoring ingredient usage levels. No. 3406. *Fd Technol., Champaign* **27** (11), 56.

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

Kligman, A. M. (1972). Report to RIFM, 23 August.

Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Moreno, O. M. (1973). Report to RIFM, 23 July.

2-ISOPROPYL-5-METHYL-2-HEXENE-1-OL

Synonym: Isodihydrolavandulol.
Structure: $CH_3 \cdot CH(CH_3) \cdot CH_2 \cdot CH{:}C(CH \cdot (CH_3)_2) \cdot CH_2OH$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By reduction of isodihydrolavandulyl aldehyde.
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·2
Maximum	0·25	0·025	0·05	1·0

Analytical data: Gas chromatogram, RIFM no. 72-66; infra-red curve, RIFM no. 72-66.

Status

2-Isopropyl-5-methyl-2-hexene-1-ol is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. 2-Isopropyl-5-methyl-2-hexene-1-ol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1973). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemical Codex (1972). 2nd Ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

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

Kligman, A. M. (1972). Report to RIFM, 25 August.

Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Moreno, O. M. (1973). Report to RIFM, 16 July.

2-ISOPROPYL-5-METHYL-2-HEXENE-1-YL ACETATE

Synonym: Isodihydrolavandulyl acetate.
Structure: $CH_3 \cdot CH(CH_3) \cdot CH_2 \cdot CH{:}C(CH \cdot (CH_3)_2) \cdot CH_2 \cdot OCO \cdot CH_3$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By acetylation of isodihydrolavandulol (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 20,000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·01	0·2
Maximum	0·25	0·025	0·05	1·0

Analytical data: Gas chromatogram, RIFM no. 72-67; infra-red curve, RIFM no. 72-67.

Status

2-Isopropyl-5-methyl-2-hexene-1-yl acetate is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. 2-Isopropyl-5-methyl-2-hexene-1-yl acetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Moreno, 1973). Tested at 10% in petrolatum it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, no. 958. S. Arctander, Montclair, New Jersey.

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd Ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

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Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Moreno, O. M. (1973). Report to RIFM, 6 July.

ISOPROPYL MYRISTATE

Synonym: Isopropyl tetradecanoate.
Structure: $(CH_3)_2 \cdot CH \cdot OCO \cdot [CH_2]_{12} \cdot CH_3$.
Description and physical properties: A colourless oily liquid.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By conventional esterification of isopropanol with myristic acid (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·02	0·5
Maximum	0·3	0·03	0·2	2·0

Status

The Council of Europe (1974) included isopropyl myristate at a level of 5 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. Isopropyl myristate is included in a provisional list of acceptable additives for use in hair-sprays compiled by the Federal Health Office of West Germany (Bundesgesundheitsamt, 1965).

Biological data

Acute toxicity. The acute oral LD_{50} value in mice was reported as >100 ml/kg (Fassett, 1963). The acute dermal LD_{50} in rabbits was reported as >5 g/kg (Moreno, 1974). When an ip dose of 100 ml/kg was given to two mice over a 4-hr period, both animals survived the 72-hr observation period (Platcow & Voss, 1954), and later the ip LD_{50} in mice was reported to be >50·2 ml/kg (Fitzgerald, Kurtz, Schardein & Kaump, 1968). Fitzgerald *et al.* (1968) also reported that the ip and sc LD_{50} values exceeded 79·5 ml/kg in rats.

Subacute toxicity. A mixture of 25% isopropyl myristate and 75% peanut oil produced only minor local damage without definitive systemic effects when injected repeatedly into rats, dogs and monkeys, or when given as single intramuscular injections to rabbits (Fitzgerald *et al.* 1968). Daily ip injection of 5 ml/kg in rats for 20 days caused three deaths after 5 days, but no growth depression or histopathological changes were observed in the surviving five rats (Platcow & Voss, 1954).

Irritation. Daily cutaneous application of isopropyl myristate to the skin of mice and rabbits for up to 28 days induced a prompt skin response (Fitzgerald *et al.* 1968). This was characterized at first by erythema, and later by lichenification and fissure formation. Histologically, acanthosis, para- and hyperkeratosis, focal erosion and focal haemorrhage were seen. In rabbits, the skin lesions regressed slowly after cessation of treatment, while in mice the lesions tended to regress during continued treatment. Similar reactions occurred with combinations of isopropyl myristate and peanut oil, but the intensities of the dermatoses were generally related to the proportion of isopropyl myristate in the mixture. Peanut oil alone produced only mild gross and microscopic changes (Fitzgerald *et al.* 1968). No signs of allergic or other types of sensitivity were observed in tests using intracutaneous injection in guinea-pigs (Platcow & Voss, 1954). Direct contact with the rabbit eye produced only mild, transient irritation (Platcow & Voss, 1954). No parenteral irritation was observed after injection of trypan blue solution following intracutaneous injection of isopropyl myristate into the abdominal skin of the rabbit (Platcow & Voss, 1954).

In a 48-hr occluded patch test using 20% isopropyl myristate in petrolatum on human subjects, no irritant effects were produced (Kligman, 1974), and isopropyl myristate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1974).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers using 20% isopropyl myristate in petrolatum. No sensitization reactions were produced (Kligman, 1974). Bergwein (1964) describes isopropyl myristate as being completely free of dermatological objections and possessing no sensitizing properties at all.

Metabolism. Higher molecular weight aliphatic esters are thought to be readily hydrolysed to the corresponding alcohols and acids which are then metabolized; isopropyl myristate is undoubtedly hydrolysed to normal metabolic products (Fassett, 1963). When myristic acid (as the ethyl ester) was fed to dogs, <2% of the amount fed was detected as unabsorbed material in the faeces, and no increased amount of ether-soluble acids was found in the urine (Weitzel, 1951).

Isopropyl myristate was found to be utilized as the sole source of carbon by micro-organisms (12 of 23 strains of bacteria, all of 25 strains of yeast and all of 17 strains of fungi) isolated from cosmetic products (Yanagi & Onishi, 1971).

Carcinogenesis. Isopropyl myristate (as a 50% solution in isopropyl alcohol) significantly accelerated the carcinogenic activity of 0·15% benzo[*a*]pyrene on the skin of C3H mice, and also significantly depressed the response of sensitized guinea pigs to 2,4-dinitrochlorobenzene challenge in the same way as known accelerators (Horton, Van Dreal & Bingham, 1966). No tumours were

produced when isopropyl myristate as a 1% solution in acetone was painted once weekly for 18 months on the skin of mice (Giles & Byron, 1968). The potential carcinogenicity and toxicity of isopropyl myristate was studied in female Swiss mice by the administration of repeated applications on the skin for the life-span of the animals (Stenbäck & Shubik, 1974). Tumours seen in both control and treated animals were mainly lymphomas, haemangiomas of the liver and lung adenomas, but tumours of other organs also occurred. No statistically significant increase in tumour incidence was caused by isopropyl myristate. Skin lesions, slight inflammation and ulceration were seen, but no persistent cutaneous abnormalities occurred. A few skin tumours were seen in treated areas as well as in untreated areas and in control animals. Thus a carcinogenic or toxic potential which would affect the use of isopropyl myristate in man was not detected (Stenbäck & Shubik, 1974).

Skin penetration. Isopropyl myristate was used as a solvent in studying the effect of intracutaneously injected alcohol on capillary permeability in rabbit skin, by measuring the extravasal leakage of Evans blue injected iv (Suzuki & Arai, 1966; Suzuki & Motoyoshi, 1965). Gelled isopropyl myristate was found to exert a much greater effect than petrolatum USP on the penetration of dexamethasone through stripped human skin, although it apparently had little influence on penetration through intact skin (Dempski, Portnoff & Wase, 1969).

Micro-organisms. The toxicity of isopropyl myristate to micro-organisms has been studied because of the use of this ester as a solubilizing agent in sterility testing of ophthalmic ointments. Tsuji, Stapert, Robertson & Waiyaki (1970) found that isopropyl myristate was more toxic to gram-negative than to gram-positive micro-organisms, and that *Pseudomonas aeruginosa* was the most sensitive to isopropyl myristate of the gram-negative micro-organisms tested. The toxic compounds can be removed and the toxicity of both filter- and heat-sterilized isopropyl myristate can be reduced by basic alumina treatment. The toxic effect may be due to trace amounts of acidic catalysts remaining after production of isopropyl myristate (Tsuji & Robertson, 1973).

Pharmacology. Isopropyl myristate is used in pharmaceutical preparations because it improves solubility and increases absorption through the skin. External uses include a non-irritating iodine preparation for disinfecting the skin (Powers & Rieger, 1963) and aerosol bactericidal preparations for feminine hygiene use without irritation of the skin and mucous membranes (Geistlich, 1970; Watson, 1969). Preparations for internal use include oral steroid formulations (Hirata, 1970) and anaesthetic injection solutions (Davis, Pearce & Connor, 1972).

Veterinary medications containing isopropyl myristate include oral or parenteral compositions for lungworm infections (N. V. Philips' Gloielampenfabrieken, 1964) and a spray formulation for bovine udders to treat mastitis, combat infection and improve the general skin condition (Kraus, 1965). Isopropyl myristate has been found to be an effective repository vehicle for im injection of penicillin in rabbits and for sc administration of oestrogens in ovariectomized rats (Platcow & Voss, 1954).

In assays on human forearms, vasoconstrictor activity of ointment preparations containing 0·025% betamethasone 17-benzoate in white soft paraffin was increased by the presence of isopropyl myristate (Pepler, Woodford & Morrison, 1971). Donovan, Ohmart & Stoklosa (1954) noted that the good solvent properties of isopropyl myristate might increase the therapeutic activity of formulations by the apparent alteration in particle size of the active ingredients, so that further evaluation and clinical study would be necessary before its use in extemporaneous compounding could be recommended. Studies in which the antifungal activity of paraben esters solubilized by surfactants was decreased by isopropyl myristate (Matsumoto & Aoki, 1962) indicate that the effectiveness of medicinal substances may be influenced by the presence of surfactants and oily ingredients such as isopropyl myristate.

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ISOPULEGOL ACETATE

Synonym: 1-Methyl-4-isopropenylcyclohexan-3-yl acetate.
Structure: $CH_3 \cdot C(CH_2) \cdot C_6H_9(CH_3) \cdot OCO \cdot CH_3$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By acetylation of isopulegol.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·12
Maximum	0·2	0·02	0·05	0·8

Analytical data: Gas chromatogram, RIFM no. 72-173; infra-red curve, RIFM no. 72-173.

Status

Isopulegol acetate 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 isopulegol acetate in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Russell, 1973).

Irritation. Isopulegol acetate tested at 8% in petrolatum produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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ISOSAFROLE*

Synonyms: 3,4-Methylenedioxy-1-propenylbenzene; 4-propenylcatechol methylene ether.
Structure: $CH_3 \cdot CH:CH \cdot C_6H_3 \cdot O \cdot CH_2 \cdot O$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: The *trans* form occurs in the essential oil of ylang-ylang. It has also been identified in the oils of *Illicium religiosum* and *Ligusticum acutilobum* Sieb. and Zucc. (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Preparation: From safrole by treatment with potassium or sodium hydroxide in the dry state or alcoholic solution, under pressure or at atmospheric pressure (Arctander, 1969).
Uses: In public use before the 1920s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·12
Maximum	0·2	0·02	0·05	0·8

Analytical data: Gas chromatogram, RIFM no. 72-29; infra-red curve, RIFM no. 72-29.

Status

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

Biological data

Acute toxicity. The acute oral LD_{50} value was reported as 1·34 g/kg in rats and as 2·47 g/kg in mice (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1972). Four daily oral doses of 460 mg isosafrole/kg given to rats produced macroscopic liver lesions (Taylor, Jenner & Jones, 1964). Oral doses of 500 mg isosafrole/kg/day given as a 25% solution in corn oil to rats for 41 days killed eight of ten animals, while 250 mg/kg/day killed only two of ten in 34 days (Hagan, Jenner, Jones, Fitzhugh, Long, Brouwer & Webb, 1965).

Subacute toxicity. In a feeding study, 10,000 ppm fed to rats in the diet for 11 wk produced growth retardation in both sexes and macroscopic and microscopic liver changes. No rats on this dose survived beyond 11 wk of treatment (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

Long-term toxicity. In a 2-yr feeding study in rats involving groups of ten males and ten females on 1000 or 2500 ppm and of 25 males and 25 females on 5000 ppm, slight liver damage occurred at the two lowest levels but no liver tumours were seen, while primary hepatic tumours appeared at the highest level. Growth retardation in both sexes occurred at the highest level, while at the two lower levels only slight growth retardation in females was observed. An increased number of interstitial-cell tumours in the testes and an increased incidence of chronic nephritis in the kidney were observed at the highest level. Slight thyroid damage was also observed at the 5000 and 2500 ppm level (Hagan *et al.*, 1965).

Irritation. Isosafrole applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1972). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Metabolism. On oxidation, isosafrole gives rise initially to an allyl alcohol and another, unidentified, conjugated alcohol, which are further oxidized to the vinyl ketone and piperonyl acrolein, respectively. Condensation of the vinyl ketone with an amine would then lead to the formation of tertiary aminomethylenedioxypropiophenones (Mannich bases) (McKinney, Oswald, Fishbein & Walker, 1972).

Enzyme induction. Rats pretreated ip with isosafrole showed an enhanced ring- and *N*-hydroxylation of 2-acetamidofluorene by rat-liver microsomes *in vitro*. In hamsters an injection of 200 mg isosafrole/kg inhibited all hydroxylating activities of 2-acetamidofluorene except for the 7-hydroxylation of 2-acetamidofluorene (Lotlikar & Wasserman, 1972).

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JASMINE ABSOLUTE

Description and physical properties: A viscous, clear, yellow-brown liquid having the characteristic odour of jasmine. The main constituent of jasmine absolute is benzyl acetate (Guenther, 1952).
Occurrence: Found in the flowers of *Jasminum officinale* L. and other species of Jasminum (Fam. Oleaceae) (Guenther, 1952).
Preparation: By extraction of the concrete with ethanol (*Fenaroli's Handbook of Flavor Ingredients*, 1971; Naves, 1974).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	Rarely	—	0·002	0·1
Maximum	used	—	0·03	0·3

Analytical data: Gas chromatogram, RIFM no. 72-174; infra-red curve, RIFM no. 72-174.

Status

Jasmine absolute was given GRAS status by FEMA (1965) and jasmine is approved by the FDA for food use (GRAS). The Council of Europe (1974) included jasmine in the list of substances, spices and seasonings deemed admissible for use with a possible limitation of the active principle in the final product.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1972).

Irritation. Undiluted jasmine absolute applied to the backs of hairless mice and swine (Urbach & Forbes, 1973) or to intact or abraded rabbit skin for 24 hr under occlusion (Moreno, 1972) was not irritating. Tested at 3% in petrolatum, jasmine absolute produced no irritation after a 48-hr closed-patch test on two different panels of human subjects (Kligman, 1972 & 1973).

Sensitization. In a maximization test (Kligman, 1966; Kligman & Epstein, 1975) carried out on 25 volunteers, the material (RIFM no. 72-3-174), tested at a concentration of 3% in petrolatum, produced sensitization reactions in two of the test subjects (Kligman, 1972)*. In a second maximization test (Kligman, 1966; Kligman & Epstein, 1975), carried out on 25 new volunteers, the material (RIFM no. 72-3-174R) was again tested at a concentration of 3% in petrolatum and produced no sensitization reactions (Kligman, 1973).

Phototoxicity. No phototoxic effects were reported for undiluted jasmine absolute on hairless mice and swine (Urbach & Forbes, 1973).

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*'*Spillover effect*'. In maximization testing, four unrelated materials are tested on each of 25 human subjects. In the event that one of the four test materials turns out to be a potent sensitizer (in this case it was Costus oil, which sensitized 25/25 subjects), false weak positive results may occur with the other three materials. When these three materials are subsequently retested out of the context of the serious allergen, and in the same or different groups of subjects, they prove to be negative. We refer to this as the 'spillover effect' (Björnberg, 1968; Kligman & Epstein, 1975).

JUNIPER BERRY OIL

Description and physical properties: EOA Spec. no. 113. The principal constituents of juniper berry oil include *d*-pinene, camphene, 1-terpineol-4 and other oxygenated constituents (Guenther, 1952).
Occurrence: Found in the fruit (berries) of *Juniperus communis* L. (Fam. Cupressaceae).
Preparation: By steam distillation of the dried ripe fruit.
Uses: In public use before the 1900s. Use in fragrances in the USA amounts to approximately 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·1
Maximum	0·2	0·02	0·05	0·8

Analytical data: Gas chromatogram, RIFM no. 72-175; infra-red curve, RIFM no. 72-175.

Status

Juniper berry was given GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) included juniper berry in the list of substances, spices and seasonings deemed admissible for use with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on juniper berry.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as >5 g/kg (Shelanski, 1972) and as 8·0 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Shelanski, 1972).

Irritation. Undiluted juniper berry oil applied to the backs of hairless mice and swine was not irritating (Urbach & Forbes, 1972), but applied to intact or abraded rabbit skin for 24 hr under occlusion it was moderately irritating (Shelanski, 1972). A patch test using juniper berry full strength for 24 hr produced two irritation reactions in 20 subjects (Katz, 1946). Juniper berry oil tested at 8% in petrolatum produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. No phototoxic effects were reported for undiluted juniper berry oil on hairless mice and swine (Urbach & Forbes, 1972).

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LABDANUM OIL

Description and physical properties: EOA Spec. no. 181. The main constituents of labdanum oil are acetophenone, 1,5,5-trimethyl-6-cyclohexanone and ladaniol (Guenther, 1952).
Occurrence: Found in the gum of the shrub *Cistus ladaniferus* L. (Fam. Cistaceae).
Preparation: By steam distillation of the crude gum.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·12
Maximum	0·1	0·01	0·03	0·8

Status

Labdanum was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1163). The Council of Europe (1974) included labdanum in the list of substances, spices and seasonings deemed admissible for use with a possible limitation of the active principal in the final product. The *Food Chemicals Codex* (1972) has a monograph on labdanum.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 8·98 g/kg (5·40–12·56 g/kg) (Hart, 1971). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Hart, 1971).

Irritation. Labdanum oil applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Hart, 1971). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1971). Cistus labdanum applied full strength for 48 hr in the standard occluded aluminium patch test used by the North American Contact Dermatitis Research Group did not produce any irritation or sensitization in a 62-yr-old subject with a perfume dermatitis (Larsen, 1975).

Additional published data

An extensive review of the monoterpenes in the volatile leaf oil of *Cistus labdanum* has been reported (Gülz, 1974).

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LAUREL LEAF OIL

Description and physical properties: EOA Spec. no. 119. The main constituent of laurel leaf oil is cineole (Guenther, 1950).
Occurrence: Found in the leaves of *Laurus nobilis* L. (Fam. Lauraceae).
Preparation: By steam distillation of the leaves of *Laurus nobilis* (Gildemeister & Hoffman, 1959).
Uses: In public use before the 1900s. 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	0·06	0·006	0·02	0·2

Analytical data: Gas chromatogram, RIFM no. 73-97; infra-red curve, RIFM no. 73-97.

Status

Laurel leaf oil was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.101). The Council of Europe (1974) included laurel leaf in the list of substances, spices and seasonings deemed admissible for use, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on laurel leaf oil.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·95 g/kg (3·17–4·74 g/kg) (Moreno, 1974). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1974).

Irritation. Laurel leaf oil applied undiluted to the backs of hairless mice and swine was not irritating (Urbach & Forbes, 1974), but when applied full strength to intact or abraded rabbit skin for 24 hr under occlusion it was moderately irritating (Moreno, 1974). Laurel leaf oil (RIFM no. 73-2-97) tested at 2% in petrolatum produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1974). Retested at 10% in petrolatum (RIFM no. 73-10-97R), it again produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1974). Laurel leaf oil European (RIFM no. M-10-R) tested at 10% in petrolatum produced no irritation after a 48-hr closed-patch test on two different panels of human subjects (Epstein, 1975; Kligman, 1975).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 73-2-97) was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Epstein, 1974). When retested on 25 new volunteers by the same maximization test at a concentration of 10% in petrolatum, the material (RIFM no. 73-10-97R) produced no sensitization reactions (Kligman, 1974). Laurel leaf oil European (RIFM no. M-10-R) at a concentration of 10% in petrolatum produced no sensitization reactions when subjected to this maximization test, either in a first group of 25 volunteers (Kligman, 1975) or in a second group of 24 new volunteers (Epstein, 1975). Laurel leaf oil (RIFM no. M-10-R(O)) patch-tested at 10% in petrolatum on costus-sensitized individuals produced sensitization reactions in all six test subjects (Epstein, 1975).

Contact allergy to *Laurus nobilis* L. is common in some European countries, with topical medicaments, occupational situations, clothing and food being the most common sources (Bandmann & Dohn, 1967; Foussereau, Benezra & Ourisson, 1970). Laurel leaf oil has been reported to cause hyperaemia and severe inflammation (Finkenrath, 1941; Flandin, Rabeau & Ukrainczyk, 1938; Zundel, 1936). Components of the essential oil from leaves of *Laurus nobilis* boiling above 70°C at 15 mm are said to cause an allergic reaction in human skin (Teisseire, 1966). Cross-sensitization between Frullania and *Laurus nobilis* (L.) has been demonstrated, with new results on laurel oil pointing to the possibility of a common denominator (the α-methylene-γ-butyrolactone moiety) between the laurel and Frullania (Foussereau, Muller & Benezra, 1975).

Laurel oil has been listed as a sensitizer by Fregert (1974).

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

Antibacterial properties. The essential oil from laurel was found to have bactericidal and fungicidal properties against a number of organisms. The oil was more effective than the aqueous extract from the leaves (1:10), which was not effective against *Salmonella enteritidis*, *S. typhimurium* or *Staphylococcus aureus* (Lomsadze & Pruidze, 1967).

Pharmacology. Pharmacological effects on circulation, such as the action on excised toad heart, rabbit heart, respiration and blood pressure, and on blood vessels of the hind legs of the toad were studied for various essential oils, including leaf oil from *Laurus nobilis*. The oils generally depressed the heart rate and decreased blood pressure (Haginiwa, Harada, Nakajima & Sakai, 1962).

Additional published data

Using an allergologic functional analysis, laurel leaf oil allergen has been shown to be an unsaturated ketone which can be isolated (as a mixture) with Girard reagent P (Foussereau *et al.* 1970).

Formulations including laurel oil and leaves are claimed to have antidandruff properties (Czira, 1970; Mina, 1971).

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Methods Used in the USSR for Establishing Biologically Safe Levels of Toxic Substances. Papers Presented at a WHO Meeting Held in Moscow from 12 to 19 December 1972. World Health Organization, Geneva, 1975. pp. 172. Sw.fr. 30.

While there is widespread concern about the possible hazards associated with environmental pollution both in the broad sense and in local areas where special conditions prevail, international agreement on what constitute acceptable levels of pollution is proving very difficult to achieve.

The study methods used in the USSR for determining biologically safe limits have been recorded for the first time in a specialist publication from the World Health Organization, compiled from papers presented by a group of Soviet scientists to a WHO symposium held in Moscow in December 1972. Aimed at health authorities and research workers throughout the world, the various contributions cover such topics as methods of investigation, the structure, physical and chemical properties and biological activity of harmful substances, the estimation of toxicity and evaluation of hazards, effects of long-term exposure, species and sex differences in sensitivity to toxic agents, safe levels of biological exposure and the establishment of safe levels of communal hygiene.

In many cases, the Soviet authorities, in their fastidious attention to the health implications of an expanding chemical industry, have tended to ignore both technological feasibility and economic considerations. This attitude is based on the premise that, under conditions of rapid technological progress, difficulties that appear insurmountable today will be overcome with ease tomorrow. Somewhat paradoxically, the Soviet (but not the US) authorities accept that there is a threshold for all types of harmful action, including carcinogenic and mutagenic effects. The major point of contention between the definition of the threshold limit value (TLV) adopted by the American Conference of Governmental Industrial Hygienists and the maximum allowable concentration (MAC) adopted in the USSR is that the former allows for the development of unfavourable effects and even minor occupational disease in isolated workers. By contrast, the Soviet MAC is based on the tenet that health is "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity". It follows that permissible limits for individual substances are usually lower in the USSR than those adopted in the USA; values for some chlorinated hydrocarbons differ by a factor as high as 40 or more, while five-fold differences are found in the case of some metals. Even where MAC and TLV values are identical, a measure of discordance is preserved in the difference between the concept of a mean concentration in a working area over an 8-hour period (TLV) and of a maximum concentration that must not be exceeded during the 8 hours (MAC).

There is a growing number of industrial compounds on which Soviet literature provides the sole source of toxicological information. If the present publication aids our understanding of the design, execution and interpretation of these studies, its appearance will be justified and widely acclaimed.

Developmental and Genetic Aspects of Drug and Environmental Toxicity. Proceedings of The European Society of Toxicology. Vol. XVI. Proceedings of the Meeting held at Carlsbad, June 1974. Edited by W. A. M. Duncan, L. Julou and M. Kramer. Excerpta Medica, Amsterdam, 1975. pp. vii + 332. Dfl. 98.00.

In 1974, the well-known European Society for the Study of Drug Toxicity broadened its range of interests to include all aspects of toxicity and changed its name to the European Society of Toxicology. This volume presents the Proceedings of the first meeting held by the Society after these changes had been agreed, and its four sections are compiled from papers presented at three symposia and a group of free communications.

The papers in the first section describe some aspects of the influence of age on toxic reactions. Two discuss the perinatal development of the ability to modify compounds chemically and thus to alter their toxicity. Experimental findings are considered in the light of some classical therapeutic accidents, such as the neonatal deaths that have been attributed to chloramphenicol therapy. A third paper describes the influence that immaturity of renal function in the immediate postnatal period may have on the toxicity of some chemicals, and discusses particularly the fact that some chemicals may be less nephrotoxic in neonates than in older animals because, in the former, mechanisms for concentrating foreign substances in the renal tubular epithelium are not fully developed.

The importance of genetic and sexual differences in determining the response to toxic agents is covered in the second section of the volume, while four papers devoted to environmental and wildlife toxicology form the third. Although the initial emotional impact of the conservationist lobby is now being put into proper perspective, it has left a realization of the need for careful assessment of the effects of xenobiotics on the environment. In some fields, such as pesticide manufacture, it has become not only desirable but also necessary, in order to satisfy regulatory agencies, to study the impact of chemicals on wildlife. These four papers discuss the theory and practice of such

testing, describing test systems for avian and aquatic species.

The volume is completed by a series of free communications, which occupy nearly two-thirds of the book and only occasionally relate to its central theme. Good though some of these papers are, their value here must be in some doubt, since their presence in this volume would not be suspected from its title.

As is to some extent inevitable in volumes compiled from this type of symposium, the book taken as a whole is of very variable quality. The advantage of symposia proceedings is that they often give a picture of areas in which particular progress is being made, but this does not necessarily provide the same balance of coverage as an authoritative work written to deal with a specific area of research. Many references are included with this collection of papers, however, and a good proportion of these relate to work published since 1970. Thus the contributions can serve as useful leads into research in the fields covered. In addition to the customary author index, this book has a useful subject index, a helpful adjunct frequently omitted from publications based on symposia.

Report of the Government Chemist 1974. Department of Industry: Laboratory of the Government Chemist. HMSO, London, 1975. pp. iv + 165. £2.

For some years, the Laboratory of the Government Chemist has been involved in monitoring the total diet for trace-metal contaminants, and 1974 findings are presented in detail in the above report. Mercury levels in fish were found to be slightly higher than before (0·14 ppm compared with only 0·05 ppm in 1972), increasing by about 1·5 μg the previously calculated daily intake of 5–10 μg mercury. Methylmercury accounted for 21–93% of the total mercury in fish, but the mean of 77% (obtained by exclusion of two surprisingly low values) agreed well with previous findings. Dried imported mushrooms contained very high mercury levels (mean 2·4 ppm) but the levels in other non-fish items were all below 0·1 ppm. Cadmium levels were generally low, except in beef, pork and lamb kidneys which contained on average 0·32 ppm, and in certain shellfish, whelks topping the league with a mean value of 1·6 ppm. Whelks also contained high levels of arsenic (above 15 ppm) and more than 10 ppm nickel was found in cockles. In the case of lead, herbs took pride of place (mean 2·6 ppm) and an average of 1·5 ppm was found in mussels, but levels in baby foods were all below the new legal limit of 0·5 ppm. In canned fruit and vegetables, lead and other metals tended to concentrate in the solid component. On the whole, more lead was found to dissolve from cans that were totally lacquered but had solder splashes or imperfect covering of the body seams than dissolved from plain or partially lacquered cans, apparently because in the latter the large surface area of tin acted as a sacrificial anode.

Other work of the Laboratory has involved the determination of organochlorine pesticide residues in mutton and beef fat, and a table of values obtained over the years since 1968 is presented. Somewhat disturbingly, levels of dieldrin, DDT and BHC seem to have increased in 1974 in fat samples of UK origin, a fact which receives no further comment in the report. Methods used to detect pesticides have included liquid chromatography, for separating and identifying complex mixtures, and radio-tracer studies, the latter being of value for assessing the efficiency of recovery procedures. Analytical methods for undesirable substances such as aflatoxin and medicinal additives in animal feeds have also been assessed in connexion with EEC Directives.

The Laboratory has continued its analysis of foodstuffs for nitrosamines (*Cited in F.C.T.* 1976, **14**, 49), with the aim of identifying those foods containing more than the 1 ppb (1 μg/kg) detectable by current methods. A range of liquid foods and some PVC packs have been examined for vinyl chloride, the presence of which has been confirmed in some cases by mass spectrometry, the detection limits being 0·2 ppm in PVC and 0·01 ppm in liquid foods. Methods being developed to detect even lower levels of vinyl chloride involve its distillation from the food and collection in a suitable medium.

A Canadian report of excessive leaching of lead from soldered joints in electric kettles prompted an examination of five UK brands of kettle, but no evidence of significant leaching was found, apparently because a different type of construction was involved. Tests on ironing-board cloths backed with polyurethane foam suggested that toxic isocyanate vapours could be produced, but in such minute amounts that they were not considered to present any hazard to health. The Department was also involved in the measurement of airborne asbestos in Ministry of Defence buildings with asbestos-insulated roofs, and the levels resulting in winter from turbulence created by heating fans (and roosting crows!) were found to be sufficiently high for installation of false ceilings to be recommended. The development of new or revised methods for detecting toxic substances in air, including methods for 1-naphthylamine, di-(4-amino-3-chlorophenyl)methane, ammonia, cadmium oxide fume, chromic acid mist, formaldehyde, isophorone, inorganic lead and mercury, also proceeded during the year.

Fd Cosmet. Toxicol. Vol. 14, pp. 341-345. Pergamon Press 1976. Printed in Great Britain

BOOK REVIEWS

Toxicology. The Basic Science of Poisons. Edited by L. J. Casarett and J. Doull. Macmillan Publishing Co., Inc., New York, 1975. pp. xiii + 768. £14.50.

This book, compiled almost entirely from contributions by American authors, is intended primarily as a textbook for students of toxicology in the United States. However, it may also serve as a useful reference book, both for toxicologists and for those of related disciplines. The presentation is good, the text, diagrams and tables are well set out, references are listed at the end of each chapter and the index is adequate.

The book is divided into four sections. The first (of five chapters) gives an excellent general introduction to the subject, dealing with such aspects as the history of toxicology and its role in the modern world, indices used in the evaluation of toxicity, the interpretation of results and the metabolism of toxic substances, as well as the factors in the exposure situation, the subject exposed or the environment that may influence toxicity. Deserving of special mention, perhaps, is a table on air pollutants in the chapter on the metabolism of toxic substances; this is 41 pages long—almost a book in itself! Perhaps the most important point made in this section is that the absolute safety-in-use of a substance cannot be guaranteed—a well-established concept, but one that bears repetition; toxicological investigations provide only the data upon which reasonable predictions about the probable safety of a substance can be based. As more data become available the standards set for safety may require modification.

The second section contains eight chapters, which deal specifically with toxicology as it relates to the liver, kidney, blood and eye and respiratory, central nervous, skeletal and reproductive systems. Although some of these chapters have but a brief physiological, anatomical or clinical introduction before the discussion of relevant toxicological details, a few have an unreasonably long introduction, giving information that can be found in any appropriate textbook. The theme throughout this section relates the toxic action of the substances discussed to the systemic site of action. The chapters on the liver and the kidney, organs so much involved in detoxication, seem a little short, but the chapter on the eye gives a comprehensive account of the harmful effects of a wide range of substances on the various structures of this organ. This is an extremely well-written and informative contribution. It is pointed out that the eyeball, in spite of its small size, contains derivatives of ectoderm and mesoderm, neural tissue, avascular tissue, photosensitive pigments and a system comparable to the blood-brain barrier. These unique features give the eye a potentially high sensitivity to toxic substances.

The 12 chapters in Section 3 deal specifically with teratogens, chemical carcinogens, radioactive substances, pesticides, metals, solvents and vapours, air pollutants, food additives, toxins of animal origin, phytotoxicology, plastics and social poisons. A detailed account of each topic is supported by references to work published up to 1973 and provides a valuable general survey of relevant data. Altogether the chapter on chemical carcinogenesis lists just under 400 references, and several contributions carry nearly 200 each. As in the other sections, each chapter is contributed by a different author. Naturally the presentation of the work varies widely between chapters but all give a good introduction to their particular topic and will be of general interest to many as well as of specific use to the worker in a particular field.

The fourth section deals with the subject as it relates to the clinician, the forensic or industrial toxicologist, the veterinary surgeon and the lawyer. Much of the information in this section is inevitably concerned with American law and practice, but it makes very interesting general reading.

Here then, is an unusual book which manages to combine general toxicological information with data of a much more specific nature. Provided it can be brought up to date periodically, it should be on the bookshelves for many decades.

The Search for Pure Food. A Sociology of Legislation in Britain. By I. Paulus. Law in Society Series. Martin Robertson & Co. Ltd., London, 1974. pp. 144. £3.25.

The purity of the food we eat has always been, and still is, a contentious subject. Legislation governing the amelioration of food was initiated in earnest in 1850 and was resolved to some satisfaction with respect to the consumer 50 years later on the passing of the 1899 Sale of Food and Drugs Act, which—unlike its 1875 predecessor—carried adequate provision for mandatory enforcement.

This slim volume charts the path of this early legislation and attempts to relate the legislative changes to sociological influences on both the law makers and the law breakers. Although the framing of the laws of the land is often thought of as a clinical exercise in reason, Professor Paulus shows this to be a simplistic, untenable view. Altogether, this is an interesting text, whose author has succeeded handsomely in giving an informative social perspective to these early food laws, although he has written a book that is likely to be of limited usefulness to those requiring a greater understanding of the later, and possibly more relevant, food and drug legislation.

Consumer Health and Product Hazards/Cosmetics and Drugs, Pesticides, Food Additives. Vol. 2 of The Legislation of Product Safety. Edited by S. S. Epstein and R. D. Grundy. The MIT Press, Cambridge, Mass., 1974. pp. xi + 389. £7.50.

This book and its companion volume (*Cited in F.C.T.* 1976, **14**, 148) review the legislative measures adopted in the USA for controlling potential health hazards arising from the manufacture and

use of consumer products. Volume 2 discusses the role of legislation in creating a balance between premarket assessment and post-market regulation of cosmetics, drugs, pesticides and food additives.

An opening chapter reviewing the development of the "new consumerism" movement points to a growing scepticism over the net value to society of inexorable economic growth and technological innovation. Outlining the major consumer-protection laws enacted in the USA over the last decade, the author points to the need for institutional reform that would facilitate an effective consumer input into both premarket assessment of consumer products and the regulatory judgements involving them.

A chapter reviewing the sources of legislative initiatives for dealing with cosmetics materials is followed by one describing the range of consumer problems facing the US government in relation to pharmaceuticals. The latter is little more than a chronological arrangement of Senatorial quotations and presents the reader with a formidable and somewhat unrewarding task. In a succeeding chapter, the US legislative measures on organic pesticides are reviewed with rather more success, in that the subject matter is organized into four identifiable areas of regulatory activity (agricultural, health-orientated, environmental and farm-worker protection).

A review of the principles governing food-additive regulation in the USA deserves more detailed scrutiny. The author describes the attempts of succeeding legislators to define "food safety" in practical terms and hence to minimize the hazard associated with chemicals added to the food supply. These attempts have met with varying degrees of failure, ranging from the Pure Food and Drugs Act of 1906, which contained no provisions for establishing safe quantities of added poisonous ingredients, to the stultifying ramifications of the 1958 Delaney Clause, which requires the prohibition of any additive found to induce cancer in animals or man. This well-presented and lucid review is certainly worth reading, but it is difficult to recommend as a whole a volume with such variable and immiscible contributions.

The Mammalian Kidney. Biological Structure and Function 5. By D. B. Moffat. Cambridge University Press, 1975. pp. viii + 263. £9.

Introductory textbooks on the kidney must inevitably be compared with the earlier work of Homer Smith. Since the latest edition of his small volume was published, a large body of knowledge has accumulated on the structure and function of this organ and this is reflected in the content and presentation of the present book. As the earlier work had a strong physiological bias, so this book reflects the great expansion that has occurred in recent years in the understanding of ultrastructure and especially in attempts to correlate this with function.

The chapters are based broadly on morphological divisions, although in relating structure to function there are inevitably large areas of overlap. The metabolism of protein, sodium and potassium are discussed in some detail and the important topics of interstitial tissues and the juxtaglomerular apparatus are given chapters of their own.

Obvious mistakes are few, although it is surprising to read in the discussion on the glomerular filtration rate in chapter 1 that the inulin concentrations in the plasma and final urine are the same. In general however, the text is well presented and easy to read, although the halftones of some of the electron micrographs are very poorly reproduced.

At £9, the book may seem rather expensive, but it is full of useful information and references and is therefore to be recommended.

Intestinal Absorption and Malabsorption. Edited by T. Z. Csáky. Raven Press, New York, 1975. pp. x + 308. $18.95.

Intestinal Absorption in Man. Edited by I. McColl and G. E. Sladen. Academic Press Inc. (London) Ltd., London, 1975. pp. ix + 363. £11.

These two volumes are essentially complementary. The first one cited consists largely of contributions from American authors and deals with recent advances in the biochemical and physiological aspects of the mechanisms of intestinal absorption. The predominantly British contributions in the other are concerned mainly with intestinal absorption in man, in health and disease. Together they present an up-to-date account of the subject as a whole, and each is well endowed with adequate references and a subject index.

The general format of each volume is similar, in that both contain papers dealing specifically with the absorption of the major categories of nutrients, although in the book edited by T. Z. Csáky there is no specific paper on lipids. In fact the term 'lipid' does not even appear in the index. The two volumes are also similar in that each topic is presented as a synopsis of the present state of knowledge, accompanied in most cases by some illustrative data usually drawn from work published by the author.

In any compilation of essays from a number of contributors, individual contributions differ considerably in terms of detail, clarity and comprehensiveness. Thus in *Intestinal Absorption and Malabsorption*, one might have anticipated a more comprehensive survey of epithelial transport phenomena by H. H. Ussing, in view of his status in the field. Moreover, the small chapter on peptide transport by D. M. Matthews could well have been expanded, although a possible explanation of this may be the recent publication of a book on the subject by this author and J. W. Payne.

Some duplication of information between the two volumes was inevitable. Clearly in the volume on *Intestinal Absorption in Man*, it would have been impossible to present a coherent account without reference to animal data, most of which appears also in the other volume. Nevertheless, the effort that has been made to highlight the human data on absorption has been successful.

For those working in the field of gastro-enterology, irrespective of whether their experimental approach involves *in vitro* methods, or animal or human investigations, these two volumes will be of considerable value. Each is the work of writers who have made notable contributions to their subjects.

Recent Advances in Pathology. No. 9. Edited by C. V. Harrison and K. Weinbren. Churchill Livingstone, Edinburgh, 1975. pp. ix + 279. £9.50.

Scientists from other disciplines often relegate pathology to the group of sciences that advance at a snail's pace, if at all. The nine contributions in this volume should go some way towards dispelling such a view, providing as they do a glimpse of the new observations made and the many new ideas generated in the last 10 years.

These advances are particularly evident in the section on the lymphocyte, a chapter that not only reviews the progress made in the understanding of the functions of this widely distributed type of cell but also gives an indication of the role it may play in various pathological processes. Unfortunately there is little material here for the histologist; one might have expected a more detailed description of the histology of organs such as the thymus, lymph nodes and spleen, which are involved in the manifestation of immunological disorders. The chapter on lymph-node diseases and diseases of the liver, on the other hand, provides a most useful guide for histological diagnosis. Both of these chapters are clearly written and important diagnostic points are emphasized. They provide good examples of the changes in and additions to histological classifications that have been made possible by improvements in our understanding of disease processes. Three other sections to which this point applies are those on medullary carcinoma of the thyroid, on miscellaneous soft-tissue sarcomas and on the pathology of the innervation of skeletal muscle. These topics are much more restricted in scope than the first two mentioned here and the authors have taken advantage of this limitation to discuss their subjects in a fair amount of depth.

A section on amyloidosis is particularly valuable. The advances in this field have been mainly in connexion with the chemical composition and ultrastructural configuration of amyloid and, because of the diverse disciplines involved, important information has tended to be published in journals not readily available to the average pathologist. The author has succeeded in bringing together enough of this information to provide a concise, comprehensive and up-to-date account of this baffling topic.

The book achieves the objectives set forth by the editors. In most sections emphasis is laid on the histological manifestations of disease and sufficient fundamental information and theory are given to allow the histopathologist at the bench to present a mature and informed opinion. The authors themselves are widely experienced in the subjects on which they have written. Many of them are either current or past members of the teaching staff of the Royal Postgraduate Medical School, London, a balance that may seem somewhat surprising in view of the number of potential contributors from other equally eminent medical schools.

Freeze-etch Histology. A Comparison Between Thin Sections and Freeze-etch Replicas. By L. Orci and A. Perrelet. Springer-Verlag, Berlin. 1975. pp. ix + 168. DM 145.

This beautifully-produced book is not another atlas of ultrastructure. It sets out to guide (or perhaps lure) biological research workers into considering an alternative technique to the conventional ultrathin sectioning used in transmission electron microscopy. The complex sequence of fixation, dehydration, staining and plastic embedding, which is essential before a thin section can be prepared, is bound to cause modification or even complete loss of some cell components.

Put simply, freeze-etching aims to look at the ultrastructure of unfixed, unstained, unembedded, unsectioned tissue that has not been 'mucked about'. The technique involves freezing fresh (or lightly fixed) tissue quickly to -190°, sometimes in the presence of agents that limit ice-crystal growth. The piece of tissue, maintained at -190°, is mounted in a bell-jar from which nearly all the air is exhausted. A glass or metal knife-edge is moved quickly through the specimen, fracturing rather than cutting it. The fracture surface, which seems to pass preferentially along the planes of subcellular membranes, is etched by the sublimation of ice in the high vacuum (10^{-6} mm Hg). A thin film of platinized carbon is evaporated on to the etched surface, forming a stable, thin replica with three-dimensional detail. The tissue is thawed and removed chemically, leaving the replica which, being electron-transparent, is suitable for immediate examination in the electron microscope.

Though freeze-etching started in 1961, it has not yet taken firm root in the literature. Perhaps this is because the fracture plane cannot in any way be controlled. Moreover, removal of the replica involves destruction of the tissue, so that 'serial sections' are impossible.

Advocates of the method claim that it is the only way of looking at the ultrastructure of 'unaltered' cells. It has even been said, of yeast cells at any rate, that after thawing to release the replica, the uncleaved cells are able to divide and grow normally when put back into nutrient medium. However, to obtain the superb results illustrated here, the authors have resorted to aldehyde fixation and glycerol cryoprotection.

At this point, it may be appropriate to point out the differences between freeze-etch micrographs and scanning electron micrographs, such as those appearing in *Scanning Electron Microscopy in Biology. A Students' Atlas on Biological Organization* by R. G. Kessel and C. Y. Shih, reviewed recently in these pages (*Cited in F.C.T.* 1976, **14**, 149). Freeze-etching uses transmission electron microscopy to examine thin replicas of the insides of tissues. Scanning electron microscopy looks almost exclusively at the surfaces of solid objects and uses the secondary electrons emitted by the objects to form an image. Both techniques produce 'three-dimensional' pictures of great aesthetic appeal, but the types of information gained are quite different.

The book begins with a brief description of methods, a short bibliography, and an introduction to the interpretation of the structures seen. The next section, entitled "Differentiations of the cell surface in freeze-etching", is a collection of 20 flawless electron micrographs, each a full 25 × 20 cm. Opposite every micrograph is a 100–200 word descriptive guide, outlining artefacts and pitfalls as well as novel structural details. Many of the micrographs depict the characteristic 'signatures' of different membrane types,

especially the membranes covering endothelial cells during endocytosis and exocytosis. Basal infoldings, microvilli and intercellular junctions are also described.

The remainder of the book consists of pairs of electron micrographs, one thin-section micrograph facing each freeze-etch picture. They illustrate and compare the details of representative cells from the digestive tract, pancreas, liver, respiratory tract, kidney, muscle, nervous system, connective tissue and blood. Each pair is described in detail and about half of the legends end with selected references.

The authors have gone to great lengths to ensure that the free-etch and thin-section micrographs match one another pictorially as closely as possible, so that the relatively unfamiliar freeze-etch images are self-explanatory when compared with their opposite numbers. The quality of the photography and block-making is awesome—not a scratch-mark, hair or flaw appears anywhere.

I wish I could afford the $62!

Chemical Carcinogenesis Essays. IARC Scientific Publications No. 10. Edited by R. Montesano and L. Tomatis. International Agency for Research on Cancer, Lyon, 1974. pp. xiv + 230. Sw.fr. 50 (available in UK from HMSO).

The collected essays and discussions in this book formed the basis of a workshop convened by the IARC and the Catholic University of Louvain in December 1973. The aim of the meeting was to discuss experimental models that would assist in making the extrapolation of experimental carcinogenesis data from animals to man more reliable. Three major topics were covered, namely the metabolism of carcinogens, carcinogenesis *in vitro* and mutagenesis.

The initial essays in the book discuss the comparative metabolism of several carcinogens, including the aromatic amines, nitrosamines and polycyclic hydrocarbons and their epoxides, emphasis being attached to the marked species differences observed in both the pathways and rates of metabolism. The further complication of intraspecies variation in carcinogen metabolism is illustrated by reference to individual differences of benzopyrene-hydroxylase activity in man.

Papers on carcinogenesis *in vitro* deal with mammalian cell models in which carcinogenic potency is indicated by the ability of a chemical to induce cell transformation. Only fibroblastic cells have proved 'transformable', and the limited responses of cultured epithelial cells to carcinogens is emphasized in an essay on the successful maintenance of liver cells *in vitro*.

Seven essays on the wide topic of mutagenesis complete this collection of papers. Mutagenic test systems ranging in complexity from bacteria to mammals are discussed, as is their tendency to produce conflicting results. In the final analysis, these results have to be interpreted in terms of the carcinogenic potential of the mutagen, and one essay is devoted to this difficult extrapolation. The overwhelming consensus of opinion expressed by the authors is that none of the test systems discussed can stand alone as a reliable indicator of carcinogenic potential. It is clear that, in general, we are still at a very early stage in attempts to extrapolate any of these studies to the human situation.

The book suffers basically from an attempt to cover too wide an area of experimental techniques, and the consequent paucity of analytical discussion is a significant failing. One would have liked to see the main priorities for future work highlighted, and an overall synthesis of current work presented. Unfortunately the reader is left with an excess of experimental detail, and an impression that we are only just beginning to realize the limitations of the systems described and the complexities of extrapolation.

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Fd Cosmet. Toxicol. Vol. 14, pp. 347–354. Pergamon Press 1976. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

VINYL CHLORIDE—PART I

Workers involved in the production of polyvinyl chloride (PVC) are exposed to differing levels of vinyl chloride monomer (VC), the greatest exposure being in the cleaning of polymerization autoclaves. Such exposure can lead to acro-osteolysis (*Cited in F.C.T.* 1973, **11**, 1155), sometimes called 'VC disease', and to angiosarcoma (*ibid* 1975, **13**, 275). Less severe exposure to VC can occur during subsequent processing of PVC and although clinical changes may not be manifest, pathological tests detect effects characteristic of clinically advanced cases (Lange *et al. Ann. N.Y. Acad. Sci.* 1975, **246**, 18).

Acro-osteolysis

In an ill-defined survey of workers exposed to VC in Japan (Sakabe, *ibid* 1975, **246**, 78), 49 out of 1597 complained of finger trouble, mostly paraesthesia (numbness and tingling) and pain. X-ray examination of the fingers of 1599 workers revealed abnormal findings in 104 cases, 80 of which could be attributed to causes unrelated to VC exposure and only two of which were clear cases of acro-osteolysis. More thorough examinations have been carried out on 70 patients who had been employed in a PVC-production plant for 0·5–21·7 years (average 7·7 years) in various processes including autoclave cleaning (Veltman *et al. ibid* 1975, **246**, 6). Of the initial symptoms reported, upper abdominal complaints were the most common (in 42 out of 70), followed by tiredness, dizziness, increased perspiration and numbness, tingling and sensation of cold in fingers and/or toes. A survey by mailed questionnaire carried out on workers in a PVC-production plant (Spirtas *et al. Am. ind. Hyg. Ass. J.* 1975, **36**, 779) revealed dose-response patterns for all these symptoms except abdominal complaints, which were not investigated.

Veltman *et al.* (*loc. cit.*) tabulated their pathological findings, the most frequent effects being thrombocytopenia (lack of platelets in the blood) in 81%, increased bromsulphthalein (BSP) retention (indicating hepatic dysfunction) in 67%, enlargement of the spleen in 57% and reticulocytosis (indicative of active blood regeneration) in 41%. External changes consisted of club-like swelling and shortening around one or more of the terminal finger bones, accompanied by watch-glass-like nails and shortening of the nail plates, and of scleroderma-like changes of the skin of the knuckles and forearm. With some of these changes, complete regression could be expected provided that a certain degree of severity was not exceeded.

Histological findings showed hyperkeratosis, an increase in subepidermal capillaries and a lymphocytic infiltrate. The collagen bundles were swollen and the elastic fibres were thin, fissured and fragmented. This last finding distinguished the diagnosis from that of systemic sclerosis. In clinically changed skin, the histological findings were always present, but in six out of 28 cases they were found even in clinically unchanged skin, although this showed no direct correlation with the severity of internal findings.

Changes in the bones were noted as a band-like acro-osteolysis, particularly at the base of the finger nail. In most cases, complete restitution occurred after removal of the patient from VC exposure. Vascular changes were closely connected with the bone changes, and ranged from slight narrowing of the vascular lumina in the digital arteries to complete absence of filling of the arteries along the whole length of fingers affected by acro-osteolysis, the degree of severity being related to the severity of the bone changes.

As mentioned above, these authors noted a high incidence of thrombocytopenia, presenting what was apparently the first report of this occurrence in connexion with VC exposure. Slight to severe cases were found in 81% of all persons examined and values ranged from 17,000 to 143,000 platelets/μl (lower norm, 150,000/μl). There was, however, no clear-cut correlation between the platelet count and either the period of exposure or enlargement of the spleen. The authors suggest that a platelet count should be an obligatory test in PVC workers. Improvement of platelet counts occurred in only six of 29 patients who had been removed from VC exposure for 1–1·5 years.

Two specific cases of acro-osteolysis have been reported in workers exposed to VC for 2 years, only the second year being at a high level of exposure (Walker, *Proc. R. Soc. Med.* 1975, **68**, 343). Both men developed acute attacks of Raynaud's phenomenon (spasm of digital arteries, blanching and numbness of fingers), together with lytic bone lesions and thickening of the skin. After removal of these workers from exposure, the skin lesions appeared to regress and the lytic lesions to heal, but the vascular disorders seemed more permanent. In a subsequent discussion, it was stated that there was no evidence that any catalysts or additives used in the VC polymerization process were capable of producing this syndrome.

Investigations have also been carried out in PVC-processing industries (Lange *et al. loc. cit.*), and although clinical manifestations such as scleroderma-like skin changes or Raynaud's syndrome were not observed, pathological findings in laboratory tests

were similar to those in workers in PVC-production industries, where exposure to VC is generally considered to be greater.

Relationship between VC exposure and angiosarcoma

An association of PVC manufacture with the incidence of haemangiosarcoma was first suspected as a result of several deaths in the industry from this otherwise very rare type of liver tumour, but criticisms have been levelled at a subsequent analysis of 161 deaths in two VC–PVC plants showing that the number of deaths due to all cancers was 50% higher than that in a comparable but unexposed population group (*Cited in F.C.T.* 1975, **13,** 276). Because up to threefold regional variations in cancer incidence are known to occur, it was suggested that the data from VC plant workers should have been compared with local and not national mortality data (Purchase & Williamson, *Lancet* 1974, **II,** 591). It was also pointed out (Falk *et al. ibid* 1974, **II,** 784) that 135 out of the 161 deaths stemmed from a plant in Louisville, Kentucky, concerned additionally with synthetic rubber production, and that a large proportion of the workers were not directly exposed to VC, thus raising problems of interpretation of the results.

Further support for these criticisms has been offered by a study of age-standardized mortality rates for a population of 2100 male workers in Penarth, Glamorgan, exposed to VC for periods of up to 27 years (Duck *et al. ibid* 1975, **II,** 1197). In the 136 deaths in men under 75 years of age, no cases of angiosarcoma were identified within the study period, although one has occurred since then. Observed and expected frequencies of death from various causes in the whole population of workers corresponded closely, and the frequency of neoplastic disease was similar in all subgroups of the population. A subsequent report disclosed that eight of the 27 angiosarcomas diagnosed in Holland since 1950 were of the liver, but none of the 27 patients had had any traceable contact with VC (Dalderup *et al. ibid* 1976, **I,** 246).

Conclusive epidemiological studies are not possible at present owing to difficulties in tracing all individuals exposed to VC, to the long latent periods of occupationally induced cancers combined with the relatively recent use of VC, and to a lack of information on levels of exposure. However, the urgency of the situation has prompted several other cohort studies of VC–PVC workers, and their results indicate support for the results of the Louisville study (*Cited in F.C.T.* 1974, **13,** 276).

A study of relatively long-term effects (Nicholson *et al. Ann. N.Y. Acad. Sci.* 1975, **246,** 225) involved 257 individuals, each of whom had been exposed to VC for at least 5 years between 1946 and 1968. Of the 255 individuals traced, 24 had died (compared with 19 expected) and of the nine deaths due to cancer (3·9 expected) three were due to angiosarcoma of the liver. The angiosarcomas all occurred in individuals who had worked since before 1951 in PVC production, suggesting that as the time from exposure increases in cohort members with similar exposures, additional cases may occur.

The authors of another study (Ott *et al. Archs envir. Hlth* 1975, **30,** 333) were able to relate tumour incidence to levels of VC exposure. Of 594 cohort members, 72 were exposed to arsenic (which is known to produce an increased cancer risk) as well as to VC, and seven of the ten deaths in this group were due to malignant neoplasms (1·9 expected). Of the 522 individuals exposed to VC alone, 79 had died (89·1 expected), 13 as a result of malignant neoplasms (16 expected). No deaths due to angiosarcoma were observed. Although the number of deaths from malignant neoplasms was small, nine out of 13 were observed in individuals exposed to high levels of VC (4·6 expected) while only five (9·4 expected) were observed in all other groups.

These authors recognized that an increase in the time from initial exposure and in the number of individuals involved would improve the reliability of the epidemiological data, but in the interim their results are supported by four specific cases of angiosarcoma. In Sweden, Byrén & Holmberg (*Ann. N.Y. Acad. Sci.* 1975, **246,** 249) diagnosed angiosarcomas in two VC–PVC plant workers exposed to VC for 18 and 23–27 years respectively. Since both these cases were initially classified as primary liver cancers, the real number of angiosarcomas associated with VC–PVC production may be larger than has hitherto been observed. Two further cases were reported among autoclave cleaners in Germany by Lange *et al.* (*loc. cit.*) who noted that the case histories contained no information on 'VC disease'. Although evidence shows that the incidences of both acro-osteolysis and angiosarcoma are related to VC exposure, there is no correlation between the two diseases. Of seven cases of acro-osteolysis tested, none showed abnormal liver functions attributable to VC exposure (Creech & Makk, *Ann. N.Y. Acad. Sci.* 1975, **246,** 88).

Diagnosis of VC-induced liver disease

Systematic tests carried out in an attempt to relate liver abnormality to VC exposure levels revealed two cases of angiosarcoma and nine cases of portal fibrosis in 274 PVC production workers, and two cases of portal fibrosis in 909 workers not associated with PVC production in the Louisville plant mentioned above (Creech & Makk, *loc. cit.*). Blood samples were subjected to the standard sequential multiple analysis known as SMA-12, involving determination of calcium, total bilirubin, albumin, cholesterol, total protein, alkaline phosphatase, lactic dehydrogenase, glutamic–oxalacetic transaminase, glucose, urea nitrogen, uric acid and inorganic phosphorus. Abnormalities in SMA-12 tests were followed up by a battery of liver-function tests. Those cases in which the battery showed some major abnormality were subjected to special procedures and treatment. No positive correlation was found between abnormal SMA-12 examinations and workers involved in PVC production, but the incidence of abnormal batteries amongst PVC production workers (10%) was significantly higher than that in workers in other areas of production (4·1%).

The efficiency of the normal SMA-12 in locating liver abnormality has still to be determined, particularly in the light of evidence from a patient who was investigated 2·5 years after being removed from VC exposure because of persistent liver dysfunction (Berk

et al. Ann. N.Y. Acad. Sci. 1975, **246,** 70). At the follow-up investigations, routine tests gave normal values, but histology showed the persistence and possible progression of hepatic fibrosis. Clearance studies using bilirubin, BSP, indocyanine green and cholyl glycol showed only the disappearance of cholyl glycol to be abnormal. The lack of response in many tests of hepatocellular function was attributed to the relative sparing of hepatocytes in VC-associated fibrosis.

[Part II of this review, to be published in the next issue of *Food and Cosmetics Toxicology*, will be concerned principally with the clinical and histological characteristics of vinyl chloride-induced angiosarcoma, with studies in animals exposed to vinyl chloride and with evidence relating to the mechanism of action and the metabolism of the compound.]

[H. R. Potter—BIBRA]

THE METABOLIC FATE OF POLYCHLORINATED BIPHENYLS

The occurrence of polychlorinated biphenyls (PCBs) in the biosphere and their modes of entry into the food chains of animals and man were discussed at some length in a recent issue (*Cited in F.C.T.* 1975, **13,** 574). Some types of human food have been found to be particularly liable to contamination with PCBs from packaging materials (*ibid* 1975, **13,** 577) and animal feeds may become contaminated with PCBs during production, processing or storage (*ibid* 1975, **13,** 578). Despite growing evidence that PCB contamination is receding since the industrial use of these compounds was restricted, it is still an important factor in the environment and the storage and metabolism of these compounds in different animal species, especially in those used for human food, remains of considerable interest.

Distribution and metabolism in rodents

The fate of 2,4,5,2',5'-pentachlorobiphenyl (PCBP) in mice was studied by injection of about 15 mg [^{14}C]PCBP/kg into a tail vein and by intragastric introduction in the form of an emulsion (Berlin *et al. Archs envir. Hlth* 1975, **30,** 141). The animals were killed for autoradiography between 20 minutes and 24 hours after an injection and 1–32 days after oral dosage. Others dosed orally with about 7 mg [^{14}C]PCBP/kg were used for an organ-distribution study. Most of the radioactivity left the circulation within 20 minutes and certainly within 1 hour of the injection. Peak concentrations were highest in the liver and kidney, and particularly in brown fat, which was the major reservoir of unchanged PCBP after 24 hours. Some retention also occurred in the bronchial epithelium and renal tubules, but the radioactivity disappeared fairly rapidly from other tissues. Labelled material was excreted mainly into the faeces via the bile, predominantly in the form of a hydroxylated metabolite, both free and conjugated, little unchanged PCBP appearing in the faeces.

The faeces of rats fed about 15 mg 2,2',4,4',5,5'-hexachlorobiphenyl (HCBP) overnight, however, were found by gas chromatography–mass spectrometry to contain unchanged HCBP and a methoxy derivative (Jensen & Sundström, *Nature, Lond.* 1974, **251,** 219). About 1·3 and 7% of the methoxy derivative and unchanged HCBP, respectively, were excreted in 7 days; there was no metabolite in the urine. Mice given HCBP similarly excreted the methoxy derivative in the faeces, but quail produced no metabolite. Two major metabolites in the urine of rabbits fed 1% HCBP in the diet for 7 days were found to be formed in the one case by hydroxylation and in the other by hydroxylation with dechlorination (Hutzinger *et al. ibid* 1974, **252,** 698). A third, minor, metabolite was the methoxy derivative of the second major metabolite.

Yoshimura & Yamamoto (*Chem. pharm. Bull., Tokyo* 1973, **21,** 1168) reported that rats given 3,4,3',4'-tetrachlorobiphenyl (TCBP) in oil in an oral dose of 25 mg/rat on every third day over a period of 9 days produced at least three faecal metabolites together with unchanged TCBP. All the metabolites were phenolic, and the major compound was monohydroxylated TCBP. After administration of a single dose of 25 mg, some 64% of the dose of TCBP appeared unchanged in the faeces within 14 days and was thought to consist largely of material that had remained unabsorbed, while only 3·3% appeared as the monohydroxy derivative. Again, no metabolite was detected in the urine. A different picture is given by Van Miller *et al.* (*Proc. Soc. exp. Biol. Med.* 1975, **148,** 682) for 2,5,2',5'-tetrachlorobiphenyl (TCB). Rats were given 50 mg tritiated TCB by stomach tube and after 1–14 days they were killed and their tissues were examined for radioactivity levels and histological appearance. About 66% of ^{3}H activity appeared in the faeces and 10% in the urine within 72 hours, and within 14 days the corresponding figures were 72·5 and 12·9%. Highest levels of tissue activity appeared in the adipose tissue, blood, liver and skin throughout the first 7 days; there were significant ^{3}H concentrations in the thymus, brain, lung, spleen, heart, small intestine and muscle at 24 hours, but these fell rapidly within 3 days. Most of the activity in the liver was in the microsomal fraction, and there was significant activity in the mitochondria though little in the nuclei. Six metabolites were detected, one from the urine and five from the faeces, but at least 60% of the total metabolites consisted of 3-monohydroxylated TCB. There was unchanged TCB in the faeces but none in the urine.

Technical mixtures of PCBs present complications in that the inclusion of the more highly chlorinated biphenyls increases the hazards of body storage. Curley *et al.* (*Envir. Res.* 1971, **4,** 481) gave the commercial mixtures Aroclor 1254 and Aroclor 1260 in single orally intubated doses of 1600 and 3200 mg/kg, respectively, to female rats, which were killed and examined 24 hours later. They also fed Aroclor 1254 at 1000 ppm in the diet for 98 days, and at 100 or

500 ppm for up to 252 days to both male and female rats. At the same exposure level, the storage of PCB exceeded that of DDT in other experiments. Storage of PCBs was similar in both males and females given the same dietary levels and was located principally in adipose tissue. In a study of rats given technical tetrachlorobiphenyl as Aroclor 1248 in a dose of 1 g/kg by ip injection, Benthe & Schmoldt (*Arch. Tox.* 1973, **30,** 207) achieved a PCB concentration in adipose tissue of 338 μg/g. They considered that technical tetrachlorobiphenyl is unlikely to be an environmental hazard provided it does not contain higher homologues such as penta- and hexachlorobiphenyls. Albro & Fishbein (*Bull. env. contam. & Toxicol.* (*U.S.*) 1972, **8,** 26) fed biphenyl itself and a range of chlorinated isomers containing 1–6 chlorine atoms to rats together with a marker (squalane). Single doses of 5–100 mg/kg were given and faeces were collected until no further excretion of the test compound or squalane occurred. Faecal excretion of unchanged material accounted for less than 10% of the dose given, irrespective of the degree of chlorination or the dose. This study was not, however, concerned with the possible formation and excretion of metabolites.

Male rats exposed to PCBs of low (42%) chlorine content (Pydraul A200, Monsanto Co.) in aerosol form in a concentration of about 30 g/m^3 absorbed the PCB rapidly; a liver content of 70 μg PCB/g was demonstrated after exposure for 2 hours, and 50% of this concentration was attained within the first 15 minutes (Benthe *et al. Arch. Tox.* 1972, **29,** 85). After exposure for 30 minutes the fat contained 14 μg PCB/g and brain tissue 9 μg PCB/g. There was a rapid decrease in the PCB content of the liver in the 24 hours following cessation of exposure. Levels increased in brain and adipose tissue during the first 24 hours, but by 48 hours the brain content had fallen considerably again, although the concentration in the adipose tissue had continued to rise to a maximum value of some 260 μg/g. Male rats orally intubated with 4-chlorobiphenyl or 4,4′-dichlorobiphenyl in doses of 50 mg/kg/day for 3 days excreted the 4-hydroxy and 3-hydroxy metabolites, respectively, in their urine (Safe *et al. Experientia* 1974, **30,** 720). Safe *et al.* (*Can. J. Biochem.* 1975, **53,** 415) subsequently administered the major urinary metabolite of 4-chlorobiphenyl, namely 4′-chloro-4-hydroxybiphenyl, by ip injection to rats at 50 mg/kg for three successive days, and collected urine and faeces for 1 week. The major urinary metabolite was 4′-chloro-3,4-dihydroxybiphenyl, and other urinary metabolites included 4′-chloro-3-methoxy-4-hydroxybiphenyl, 4′-chloro-4-methoxy-3-hydroxybiphenyl and 4′-chloro-4-methoxy-3,5-dihydroxybiphenyl. No metabolites of the injected compound could be isolated from the faeces and there was no evidence in either urine or faeces that oxidative fission of the biphenyl nucleus of PCBs occurred. Hydroxylation, it is suggested, may occur directly or via an arene epoxide intermediate. The biological effects and toxicity of the hydroxy metabolites of PCBs are at present unknown (Safe *et al.* 1975 *loc. cit.*).

Confirmation of the importance of hydroxylation in the metabolism of PCBs with a relatively low chlorine content has come from Greb *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1975, **13,** 424), who incubated ^{14}C-labelled 2,2′-dichlorobiphenyl, 2,4′-dichlorobiphenyl or 2,5,2′-trichlorobiphenyl with a liver-oxidase preparation from female rats which had been pretreated for 3 days with phenobarbitone to increase the activity of the enzyme system. The degree of hydroxylation achieved ranged from 10% of the dose for 2,2′-dichlorobiphenyl to 31% for the trichlorobiphenyl and 35·8% for 2,4′-dichlorobiphenyl.

Birds and fish

Pigeons were fed capsules containing tritium-labelled Aroclor 1254 in corn oil for 4 days (de Freitas & Norstrom, *Can. J. Physiol. Pharmac.* 1974, **52,** 1080). They were killed 3 days later and PCB metabolites were isolated from the lipid extracted from the omental adipose tissue. There was evidence of rapid metabolism of PCBs containing 2,3-, 3,4- or 2,3,6-substitution by chlorine on at least one phenyl ring. PCBs containing 2,4,5-, 2,3,4- or 2,3,4,5-substituents on both rings did not undergo metabolism. Compounds with the 2,5-substituents underwent metabolism far more slowly than did the 2,3- and 3,4-substituted compounds. Mobilization of PCBs from the adipose tissue of the pigeon did not appear to be affected by differences in chemical structure. In birds exposed to a period of starvation and cold, PCBs moved from adipocytes to other lipid deposits, notably muscle cells, and when normal feeding was resumed the reverse process occurred. The PCB metabolites tended, however, to be excreted rapidly and did not accumulate in adipose tissue, muscle or liver; nevertheless, they did accumulate in brain and to a lesser extent in kidney tissue, especially during the refeeding period following the period of starvation.

Hutzinger *et al.* (*Science, N.Y.* 1972, **178,** 312) studied the metabolic behaviour of pure 4-chloro-, 4,4′-dichloro-, 2,2′,5,5′-tetrachloro- and 2,2′,4,4′,5,5′-hexachlorobiphenyl in three animal species, the pigeon, rat and trout. When single doses of 460–1200 mg PCB/kg were given to brook trout, no evidence of the excretion of hydroxylated metabolites was detected in the tank water. Rats injected with 50 mg PCB/kg and pigeons given 60–100 mg PCB/kg by mouth, in both cases on three successive days, showed a similar pattern of metabolism, except that pigeons did not produce a dihydroxylated metabolite from 4-chlorobiphenyl, whereas rats produced both mono- and dihydroxylated derivatives. Both species excreted large quantities of di- and tetrachlorobiphenyls unchanged; in the rats the unchanged material was extracted from the faeces and only the monohydroxylated metabolites appeared in the urine. The hexachlorobiphenyl was excreted unchanged, no hydroxy metabolites being detected in either rat or pigeon.

Large mammals

Safe *et al.* (*J. agric. Fd Chem.* 1975, **23,** 259) injected 4-chloro- and 4,4′-dichlorobiphenyl in an iv dose of 5 mg/kg into goats and gave 4-chlorobiphenyl in a dose of 50 mg/kg orally to a cow. The first compound produced 4′-chloro-4-hydroxybiphenyl in the urine of both species and 4′-chloro-3,4-biphenyldiol in the urine of the goat. The second compound produced 4,4′-dichloro-3-hydroxybiphenyl in the goat urine. In the goats, no appreciable residues of hydroxylated metabolites remained in the heart, lung, kidney or

liver after 5 days and none were detected in the faeces. Lactating cows were fed 200 mg Aroclor 1254/day for 60 days by Fries *et al.* (*ibid* 1973, **21**, 117) and the concentration of PCBs in the milk fat were estimated. Concentrations of PCBs reached equilibrium in the milk after about 40 days, the mean concentration at 40–60 days being 61 μg/g fat. By day 60, the PCB concentration in body fat had reached 42 μg/g. When feeding of Aroclor was discontinued, the PCB concentration in milk fat fell by 50% within 15 days, and thereafter declined more slowly, being roughly parallel at this stage with the fall in the body-fat content of PCBs. Although it might be anticipated that cows with a higher milk yield would probably excrete more PCBs, no significant relationship between rate of decline of PCB in the milk fat and the amount of fat produced could be discovered.

Platonow & Geissinger (*Vet. Rec.* 1973, **93**, 287) gave Aroclor 1254 to 10-week-old piglets in single doses of 50 or 250 mg/kg by mouth and killed one male and one female from each group on days 1, 2 and 7 and at weeks 4 and 12 after treatment. In seven of the 12 pigs killed by day 7, lesions of acute hepatitis and/or cirrhosis were seen microscopically. Small cirrhotic lesions also appeared in two of the four pigs killed at week 4 and in the same proportion of those killed at week 12. Other organs showed no lesions, and no significant lesions were found in a control group. In the pigs on the lower dose, the highest tissue concentration of PCBs was found on day 1 in all tissues except the fat, in which it appeared after 2 days. By week 12, all PCB concentrations in the tissues had fallen substantially, but this was due to the growth of the animals, the total body burden of PCBs remaining nearly constant throughout the 12 weeks. In pigs on the higher dose, the proportional retention was lower, but was again roughly constant throughout the 12 weeks of the study.

In female rhesus monkeys injected iv with ^{14}C-labelled 2,4'-dichlorobiphenyl in a dose of 16·8, 77·6 or 566 μg/kg or 2,5,2'-trichlorobiphenyl in a dose of 82·6 μg/kg, about 77% of the administered radioactivity was recovered from urine and faeces within 14 days, and about 17% of the urinary metabolites were shown to be in the form of sulphate or glucuronide conjugates (Greb *et al. Bull. env. contam. & Toxicol. (U.S.)* 1975, **13**, 471). Urinary and faecal metabolites were identical, and no free unmetabolized PCBs could be detected. Studies *in vitro* provided no evidence of any involvement of the intestinal bacteria in the processes of PCB metabolism. The total conversion of these PCBs to hydroxylated metabolites and their rapid excretion indicates that, in primates, low doses of the less highly chlorinated biphenyls will not accumulate in the tissues.

[P. Cooper—BIBRA]

MORE LIGHT ON HEXACHLOROBENZENE

The increased occurrence of hexachlorobenzene (HCB) in the environment has given rise to some concern. It is used as a fungicide to control bunt of wheat and can also occur as a waste by-product of chlorinated hydrocarbon manufacture. HCB has been detected in the tissues of wild birds in the Netherlands and in adipose tissue of cattle in Louisiana (Kimbrough & Linder, *Res. Commun. chem. Path. Pharmac.* 1974, **8**, 653), and in humans in New South Wales (*Cited in F.C.T.* 1973, **11**, 913). In Turkey, an outbreak of poisoning occurred after human consumption of HCB-treated grain (*ibid* 1967, **5**, 429) and involved cutaneous porphyria with photosensitivity, hyperpigmentation and hypertrichosis, osteoporosis and enlargement of liver, thyroid and lymph nodes.

One method of environmental distribution of HCB has been described by Burns & Miller (*Archs envir. Hlth* 1975, **30**, 44), who followed up the finding that in a limited area in Louisiana adjacent to a plant producing chlorinated solvents the adipose tissue of cattle contained significant residues of HCB. Plasma HCB levels in a sample of 86 individuals living in the contaminated area, but not occupationally exposed, averaged 3·6 ppb ($b = 10^9$), with a maximum of 23 ppb. Plasma HCB concentrations were higher in males than in females (4·71 ppb compared with 2·79 ppb, respectively) but there were no significant differences between age groups. There was no evidence of cutaneous porphyria in this population, but persons with raised plasma concentrations of HCB showed abnormally high coproporphyrin and lactic-dehydrogenase levels. Only two of 48 household meals sampled contained significant quantities of HCB but in individual families there was some correlation between concentrations in plasma and in household dust. When affected households were mapped, they proved to be on the route of a truck which regularly conveyed residues containing HCB from a factory to a dump. Workers in the adjacent plant engaged in manufacturing carbon tetrachloride and perchloroethylene had plasma-HCB concentrations ranging from 14 to 233 ppb.

Toxic effects of HCB in animals

The effects of feeding technical grade (93–95%) HCB to rats at dietary levels of 100–1000 ppm for 4 months are reported by Kimbrough & Linder (*loc. cit.*). At the 1000 ppm level three of ten males and 19 of 20 females died, and at the 500 ppm level two of ten males and 14 of 20 females died. At these feed levels, liver, spleen, adrenals, lungs and kidneys were increased in weight compared with controls. Neither survival nor organ weights were affected by 100 ppm HCB, apart from a slight increase in liver weight in males. The white blood cell count was raised 30% in males fed 500 or 1000 ppm HCB, and 52% in females fed 500 ppm HCB, while haemoglobin and haematocrit values fell in females fed 100 or 500 ppm HCB and in males fed 1000 ppm HCB. Microscopic examination showed significant alteration in liver,

heart, lungs and adrenals. Liver sections showed enlarged hepatocytes, multinucleate giant cells and focal degeneration of parenchyma, accompanied either by inflammatory exudate or by fibrosis. In addition, interstitial fibrosis sometimes occurred. In rats fed the higher levels of HCB, the heart showed areas of fibrosis or muscle-fibre degeneration with inflammatory infiltrates. In the lungs increased numbers of macrophages filled many alveoli and bronchioles. Females on the higher feed concentrations developed extensive intra-alveolar haemorrhage, pulmonary oedema and sometimes an eosinophilic membranous alveolar lining and inflammatory exudate. Adrenal cortical hypertrophy was limited to the zona fasciculata, and there was occasional adrenal congestion and haemorrhage. In addition to these organ changes, occasional mild chronic nephritis and myeloid metaplasia of the spleen appeared. Ultraviolet fluorescence of the gastro-intestinal tract and liver indicated porphyria in some animals. It was not clear whether all these effects could be attributed to HCB itself, or whether trace contaminants in the technical-grade HCB (carbon tetrachloride, perchloroethylene, hexachloroethane, hexachlorobutadiene, pentachlorobenzene, octachlorodibenzofuran, decachlorobiphenyl and octachlorobiphenylene) might be implicated.

In another study (Villeneuve, *Toxic. appl. Pharmac.* 1975, **31**, 313) rats were fed 1–100 mg HCB in corn oil daily for 14 days in addition to a standard diet. Thereafter, groups were either allowed to feed *ad lib.* or were given only 25% of their normal food intake for 9 days. No animal died in the 100 mg HCB group during the 14-day dosing period, but one of five died 4 days later during *ad lib.* feeding and four of five died 3–10 days after transfer to the 25% diet. During the 2 days before death, anorexia, tremors and a reddish nasal discharge were observed. In the food-restricted group, spleen weight was increased over control values in rats previously fed 100 mg HCB/day, and liver weight was increased in rats previously fed 10 or 100 mg/day. In this group, HCB concentrations in plasma, adipose tissue and organs rose substantially after cessation of the 10 or 100 mg HCB/day treatments, and death was associated with a brain HCB concentration exceeding 300 ppm. Faecal excretion of HCB, previously shown to be the major excretion route, was only 1% of the amount administered and was not dependent on the subsequent diet. It was evident that food restriction mobilized HCB dissolved in body fat, but did not increase its excretion.

Effect on the rat foetus

Villeneuve & Hierlihy (*Bull. env. contam. & Toxicol. (U.S.)* 1975, **13**, 489) examined foetuses removed on day 22 from rats fed 5–120 mg HCB/kg/day from day 6 to 16 of gestation. Significant quantities of HCB accumulated in foetal tissue in a dose-dependent manner, the highest concentrations appearing in liver and brain. These concentrations, and the total foetal concentration, were several times lower than that of HCB in the maternal liver, irrespective of the dosage level. This finding was in contrast to the results in the rabbit, in which foetal liver concentrations of HCB were higher than those in the maternal liver and much higher than those in foetal brain (*Cited in F.C.T.* 1975, **13**, 476).

Metabolism and excretion

Liver residues of HCB were measured in male and female rats after feeding 10–160 ppm HCB in the diet for 9–10 months (Grant *et al. Envir. Physiol. Biochem.* 1974, **4**, 159) and were found to be dose-related and similar for both sexes. Body weights among females fed 160 ppm HCB were significantly less than those of the controls. The ratio of liver to body weight was raised in these animals and in males fed 80 or 160 ppm HCB. Induction of microsomal cytochrome *P*-450 activity was significant at a feed level of 40 ppm HCB in males but at no level in females. NADPH cytochrome *c* reductase activity was increased in males and females at feed levels of 40 and 160 ppm HCB, respectively. Microsomal homogenates from males fed 40 ppm HCB or more, but not from females, had increased aniline-hydroxylase and *N*-demethylase activities. However, pentobarbitone and zoxazolamine metabolism by homogenates was increased in preparations from both sexes fed 20 ppm HCB or more, thus indicating that HCB did induce mixed-function oxidases in females as well as in males. Only in females was there significant chemical porphyria.

In an attempt to establish a relationship between onset of porphyria and changes in liver mixed-function oxidases, male rats were fed a diet containing 0·2% HCB for up to 100 days (Lissner *et al. Biochem. Pharmac.* 1975, **24**, 1729). Urinary excretion of porphyrin and 5-aminolaevulinic acid increased over that of controls after day 40 of administration. Cytochrome *P*-450 levels, however, increased rapidly to three times those of controls. They maintained this level until day 30, after which they rose steeply until the end of the study. The authors proposed that unchanged HCB, possibly bound to the lipoprotein components of the endoplasmic reticulum, caused the initial microsomal enzyme induction. This could have led to the oxidation of HCB, the metabolite so formed being the porphyrogenic agent and also being a more potent inducer of the microsomal enzymes.

From a comparison of microsomal spectral changes and of rates of loss of radioactivity from ^{14}C-labelled haemoprotein of the CO-binding particles obtained from liver microsomes, Stonard (*ibid* 1975, **24**, 1959) has proposed that HCB is representative of a new class of microsomal-enzyme inducers, which share the properties of both the polycyclic hydrocarbon group (represented by 3-methylcholanthrene) and the phenobarbitone, chlordane group.

In male rats fed for 10 days on a diet containing 333 ppm HCB, the activities of aniline hydroxylase, biphenyl 4-hydroxylase, biphenyl 2-hydroxylase, 4-nitroanisole *O*-demethylase, esterase, cytochrome *P*-450 and cytochrome b_5 in microsomal homogenates of liver were all increased compared with control values (Turner & Green, *Biochem. Pharmac.* 1974, **23**, 2387). In particular, biphenyl 2-hydroxylase, 4-nitroanisole *O*-demethylase and cytochrome *P*-450 were increased several-fold. The authors suggested that the evident stimulation of 2-hydroxylation by HCB might, by increasing the production of *o*-aminophenol metabolites from some polycyclic amines, present a carcinogenic hazard.

Administration of ^{14}C-labelled HCB to male rats in a single oral dose of 5 mg/kg resulted in excretion

of 16% in the faeces and less than 1% in the urine within 7 days (Mehendale *et al. J. agric. Fd Chem.* 1975, **23,** 261). More than half the faecal excretion occurred within the first 3 days. Labelled HCB residues appeared in every major organ and tissue, with 43% of the total dose in body fat, and smaller amounts in muscle, skin and liver. On a concentration basis, the small intestine, stomach, liver and skin contained more activity than muscle. The only evidence of labelled HCB metabolites was in the urine, which contained pentachlorobenzene, tetrachlorobenzene, pentachlorophenol and four unidentified compounds. No faecal metabolites were detected. The body stores consisted mainly of HCB with traces of dechlorinated metabolites.

When incubated with homogenates of liver, lung, kidney and small intestine from control rats, [^{14}C]HCB produced trace amounts of chlorobenzene (Mehendale *et al. loc. cit.*). Co-factors were apparently not essential to this reaction. Liver microsomal preparations with added NADPH produced one or more chlorophenols. Rats pretreated with 10 or 25 mg HCB/kg/day for 6 days showed dose-related induction of microsomal cytochromes *P*-450 and b_5, glucuronyl transferase, aniline hydroxylase and ethylmorphine and 4-nitroanisole demethylases. In those rats given 25 mg HCB/kg, urinary coproporphyrin excretion was more than doubled by day 4 of treatment, remaining constant thereafter, but there were no signs of porphyria.

Thin-layer chromatography of chloroform extracts of urine from male rats fed 0·25% recrystallized HCB in the diet showed a single significant spot (R_F 0·56) matching that of authentic pentachlorophenol, while a considerable amount of polar material remained close to the origin (Lui & Sweeney, *FEBS Lett.* 1975, **51,** 225). Gas chromatography demonstrated a peak with a retention time matching that of pentachlorophenol. Rats consuming 50–100 mg HCB daily produced 12·5 μg pentachlorophenol in their daily urine output of about 15 ml. Other urinary metabolites, detected by on-column methylation, were presumed to be derivatives of HCB. Bioconversion of HCB to pentachlorophenol, which is markedly hepatotoxic, and/or the formation of reactive intermediates able to form covalent bonds with cellular constituents, were postulated as possible factors in the overall toxicity of HCB in animals.

The extrapolation of animal data to man should always be treated with caution, particularly in the case of compounds for which species-specific effects have been reported. HCB was clearly brought into this category by a recent *in vitro* study using [^{14}C]HCB (Yang *et al. Life Sci.* 1975, **17,** 545). This revealed that HCB binds to erythrocytes of rats, mice and rabbits, but that there is little or no binding to erythrocytes of 14 other species including man. Similar binding did not occur with erythrocytes either from rats or man when [^{14}C]DDT or [^{14}C]Mirex replaced [^{14}C]HCB. Further inter-species comparisons will clearly be required before the many animal studies so far reported can be confidently assessed in terms of their relevance to man.

[P. Cooper—BIBRA]

ULTRA-SHORT-TERM TESTS IN BACTERIAL SYSTEMS

Bacteria have been used for studying the mutagenic activity of chemicals for at least two decades but until recently no systematic investigations had been carried out on a scale large enough to permit any assessment of the value of such tests in predicting mutagenic and carcinogenic hazard. There are probably two main reasons for the increasing attention now being given to bacteria in this context. One is the gradual acceptance of somatic mutation as a cause of cancer by a majority of workers in this field and the other is the development of special bacterial strains that have greatly facilitated the identification of mutants.

With such special strains, a group at the University of California set about testing a variety of carcinogenic agents. Their tests, the results of which have been published in a series of papers, fall into three basic groups. In the first (Ames *et al. Proc. natn. Acad. Sci. U.S.A.* 1973, **70,** 782), several potent proximate carcinogens were tested. A subsequent publication (*idem, ibid* 1973, **70,** 2281) reported the results obtained with a range of 18 pre-carcinogens, which were inactive on their own but induced mutations when a preparation of liver microsomes was added to the incubation mixture. At a meeting held in Honolulu in December 1974, mutagenicity data on a large number of chemicals were reviewed and a high correlation was found to exist between carcinogenic activity in animals and mutagenic activity in bacteria, with about 80% of the known chemical carcinogens tested showing mutagenic activity (de Serres, *Mutation Res.* 1975, **31,** 203).

On empirical grounds a correlation of this order may seem an adequate basis for recommending bacterial mutagenesis tests as a screen for carcinogenesis, but digging a bit deeper, one begins to question whether bacterial mutagenesis is really as good as the numerical correlation suggests. To begin with, the four strains of bacteria originally used failed to give a positive result with some potent proximate carcinogens (Ames *et al. Science, N.Y.* 1972, **176,** 47; *idem, Proc. natn. Acad. Sci. U.S.A.* 1972, **69,** 3128) and the consequent need for modifications in the test procedure and strains to improve sensitivity may complicate the interpretation of data.

With the pre-carcinogens, a positive result was obtained when a microsomal preparation (the 9000 **g** supernatant of a liver homogenate) was added to the preparation (*idem, ibid* 1973, **70,** 2281). If one compares the potency of response in animal studies with the mutagenic potency in the bacterial systems used, however, one finds that some weak carcinogens, such as 2-aminoanthracene, evoked strongly positive re-

sponses, whereas some potent carcinogens, including 7,12-dimethylbenz[*a*]anthracene, yielded only weakly positive results. This inverse relationship does not, of course, invalidate the test, but it serves to bring home the fact that a wide gulf exists phylogenetically between the test system and the mammal.

There are other reasons for urging the exercise of caution in this connexion. Earlier studies showed that products of normal intermediary metabolism, such as formaldehyde and acetic acid, were capable of mutagenesis in bacterial systems (*Cited in F.C.T.* 1963, **1**, 312; *ibid* 1965, **3**, 515). The former was particularly active in bacteria possessing a repair-deficient DNA. Secondly sodium nitrite, one of the most potent mutagens in bacterial systems (Kaudewitz, *Z. Naturf.* 1959, **14b**, 528), has not induced tumours in mammalian species even when administered at dose levels close to those capable of inducing methaemoglobinaemia (*Cited in F.C.T.* 1964, **2**, 92). These examples indicate the need for a critical evaluation of results obtained in bacteria and highlight the importance of taking into account factors other than the mere production of bacterial mutants. One important factor is the wide difference in the pharmacokinetics of compounds in the mammal and in bacteria; reductive processes predominate in foreign-compound metabolism in bacteria, while oxidative processes are more important in mammals.

Another major factor is the difference between bacterial and mammalian DNA. Apart from obvious differences in levels of DNA and the shorter DNA replication cycle in bacteria compared with that in any mammalian cells, there are major differences in the repair of DNA damage. According to Painter (*Genetics* 1974, **78**, 139), DNA damage is repaired in the same general way in bacteria as in the mammal. In both bacterial and mammalian cells, repair is principally either by excision and re-synthesis or by a 'by-pass' (post-replication) process if the damage cannot be appropriately repaired by excision. However, the latter process seems to be much more prevalent in bacteria than in mammals, a difference that may have considerable significance in the interpretation of screening tests for mutagenicity. Furthermore, there is good evidence that by-pass repair in bacteria involves recombinational events between newly formed and already existing DNA (Rupp *et al. J. molec. Biol.* 1971, **61**, 25), whereas no evidence for this has been forthcoming from mammalian cells. This difference is of fundamental importance in toxicology because there seems to be sound experimental evidence indicating that the recombinations are not always successful in reproducing the original structure and it is thought that many of the mutations observed in bacteria are due to such faulty recombinations. If this is the case, and there are good reasons for accepting it, mutagenesis in bacteria is a reflection of an inherent 'error-prone' system and is no more than an indication that a particular chemical, given the right conditions, may be capable of damaging mammalian DNA. Whether such damage would then result in mutations is a totally different proposition.

There is thus strong support for the widely held view that induction of mutations in bacteria cannot be advanced automatically as evidence that mutations are likely to be caused in mammals by the particular compound in question. Evidence that bacterial mutagenesis is suggestive of carcinogenic activity is even weaker, despite the correlations demonstrated in the work reviewed above, not only because the link between mutagenesis and carcinogenesis is little more than theory but also because the list of compounds tested, although impressive, is not nearly comprehensive enough in terms of cytotoxic non-carcinogenic agents. These considerations are important if a toxicologist is faced either with assessing a compound of unknown potency on the results of bacterial tests alone or with balancing a positive result in a bacterial system with a negative result in a well-conducted test in a mammalian *in vivo* system. Most authorities seem to agree that no rational assessment of human hazard can be made on bacterial tests alone, while the question of the validity of positive bacterial results in conflict with negative results obtained for the same compound in a mammalian *in vivo* system has been brought into sharp focus by the controversy surrounding the positive results of mutagenicity tests carried out in bacteria on various constituents of hair dyes (Ames *et al. Proc. natn. Acad. Sci.* 1975, **72**, 2423). Because of the widespread use of hair dyes, these findings immediately evoked a strong reaction. Fortunately, several of the same compounds had yielded clearly negative results in well-designed, long-term carcinogenicity tests in various mammalian species (Kinkel & Holzmann, *Fd Cosmet. Toxicol.* 1973, **11**, 641; Burnett *et al. ibid* 1975, **13**, 353; Wernick *et al. Toxic appl. Pharmac.* 1975, **32**, 450). In contrast to some articles in the popular press, which tended to take a sensational line, the scientific press has taken a cool deliberate look at the problem and, although some debate is still going on and a call for more tests has been made, responsible opinion seems on the whole to have placed more weight on the animal studies than on those in bacteria (*British Medical Journal* 1975, **4**, 188; *Lancet* 1975, **II**, 218). To quote the former: "Scares based on wild extrapolations from ultra-short-term studies in artificially susceptible laboratory systems serve to confuse issues rather than to elucidate them". May this thought remain impressed on the minds of all concerned!

In the final analysis, it remains to be seen whether the genetic bases of *E. coli* and *E. lephant* are the same and whether the response of their genetic apparatus to chemical mutagens may be equated.

[P. Grasso—BIBRA]

Fd Cosmet. Toxicol. Vol. 14. pp. 355–368. Pergamon Press 1976. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

3024. Odorous metabolism

Hawksworth, G. & Scheline, R. R. (1975). Metabolism in the rat of some pyrazine derivatives having flavour importance in foods. *Xenobiotica*, **5**, 389.

In recent years pyrazines have been shown to contribute significantly to the flavours of roasted or cooked foods. The metabolism of simple alkyl- and alkoxypyrazines, many of which exhibit organoleptic properties, has not previously been reported. In the study cited here, pyrazine and pyridine derivatives were administered intragastrically to albino rats and their urinary metabolites were identified.

None of the compounds studied appeared unchanged in the urine and excretion of metabolites was completed within 24 hr. 2-Methylpyrazine was oxidized to the 2-carboxylic acid, and 2,5- and 2,6-dimethylpyrazines were oxidized to 2-methylpyrazine-5- and 2-methylpyrazine-6-carboxylic acid. The formation of acid in each case was almost quantitative. With 2,3-dimethylpyrazine, however, the aliphatic oxidation was severely inhibited and there was a considerable degree of ring hydroxylation (5-OH). The phenol was excreted as a sulphate or glucuronide conjugate.

The corresponding methyl pyridines exhibited a similar metabolic pattern, although conjugation with glycine was much more extensive, being essentially complete with the monocarboxylic acid metabolites of 2-methyl- and 2,6-dimethylpyridine. With 2,3-dimethylpyridine, aliphatic oxidation was less marked (as with the pyrazine analogue), the carboxy derivative was excreted as such rather than as the glycine conjugate, and ring hydroxylation with conjugation occurred.

O-Demethylation and ring hydroxylation occurred with both 2-methoxypyrazine and 2-methoxypyridine. In both cases, the *O*-demethylated compound accounted for some 20% of the metabolites found. The exact structure of the sulphate- or glucuronide-conjugated phenols was not determined. An attempt to effect *O*-demethylation of 2-methoxypyrazine by anaerobic incubation with micro-organisms from the rat caecum proved unsuccessful.

The study also showed that little or no biliary excretion of the pyrazines or their metabolites took place.

In view of the relatively simple metabolism of these model pyrazines, the metabolism of 2-isobutyl-3-methoxypyrazine, the component responsible for the characteristic flavour of bell pepper, was studied. Again *O*-demethylation occurred, giving as the major metabolite 2-isobutyl 3-hydroxypyrazine, which was partly excreted in unconjugated form. Aliphatic side-chain oxidation also occurred to a small extent, but confirmation of the structure of the resulting acid(s) was not possible.

3025. Maternal alcoholism and its effects on pregnancy

Tze, W. J. & Lee, M. (1975). Adverse effects of maternal alcohol consumption on pregnancy and foetal growth in rats. *Nature, Lond.* **257**, 479.

Much work has been carried out on the effects of ethanol on reproductive performance and it has been shown that, under certain conditions, ethanol can act as a mutagen (*Cited in F.C.T.* 1975, **13**, 581). The study cited above was designed to provide a simple experimental model to elucidate some information on the adverse effects of maternal alcoholism.

Female rats were divided into two test groups and one control. For a 5-wk period prior to mating, one group (A) received ethanol as a 30% aqueous solution together with a balanced powdered diet, group B received water *ad lib.* and a diet providing a calorie intake equal to that derived by group A from both the powdered diet and ethanol, and a control group (C) received food and water *ad lib.* After mating, group A animals continued to receive alcohol, and the intake of group B animals was regulated accordingly during gestation. After an initial sharp decline in weight, animals of group A gained weight in a pattern similar to that of groups B and C, but their mean weight remained at least 20 g below those of the latter groups. While the incidence of pregnancy did not differ significantly between the three groups, only 50% of the group A animals known to have copulated delivered litters, compared with 88 and 91% in groups B and C, respectively. The average litter size was significantly lower (7·2) in group A than in groups B and C (10·5 and 11·7, respectively). The mean birth weight was also lower in group A than in groups B and C (4·58 g compared with 6·0 and 5·88 g, respectively). No significant variation was found in the gestation periods recorded in the three groups. Offspring of group A exhibited microcephaly, a cracked, dry and loose skin, reddened areas on the head and body and a generally shrivelled appearance.

These figures demonstrate that, in the rat, alcohol consumption before and during pregnancy decreases significantly the size and number of progeny produced. These effects are not related to calorie intake, but appear to be due directly to the ingestion of alcohol.

MISCELLANEOUS DIRECT ADDITIVES

3026. Iodine hazard for the goitrous?

Stewart, J. C. & Vidor, G. I. (1976). Thyrotoxicosis induced by iodine contamination of food—a common unrecognised condition? *Br. med. J.* **1**, 372.

> "Bread and milk have traditionally been regarded as the ultimate in innocuous fare. In the present circumstances it may be that for the goitrous cakes and ale provide safer sustenance."

Iodine-induced thyrotoxicosis reported prior to the study cited above has been ascribed either to clinical administration of iodine or to deliberate iodine supplementation of the diet to combat endemic goitre; the thyrotoxicosis epidemic in Tasmania in 1966 was attributed to the latter cause. The authors cited above report additional, although less severe, increases in the incidence of thyrotoxicosis in Tasmania in 1964 and 1971 and relate these to the contamination of milk by iodine.

Iodine-induced thyrotoxicosis seems to occur only when the thyroid is unresponsive to control by the pituitary. Unresponsive thyroid tissue is found in Grave's disease and in nodular goitre. It is also present in some goitres that occur in iodine-deficient areas. Administration of iodine in such instances results in an excessive production of thyroid hormone and the appearance of symptoms of thyrotoxicosis. Thus successive increases in iodine intake may cause successive waves of thyrotoxicosis, such as were observed by these authors.

The numbers of thyrotoxic patients seen in hospitals in Northern Tasmania between 1960 and 1974 were taken to reflect the actual incidence of the disease. The incidence of thyrotoxicosis during 1960–63 was stable, but in 1964 and 1965 there was a significant ($P < 0{\cdot}02$) increase, preceding the more dramatic increase that began late in 1966, the year in which iodate was first added to the island's bread. After 1967 the incidence declined, except in 1971 when the trend was temporarily reversed for patients over the age of 40 yr.

The effects of using iodine-containing additives in continuous-mix automated baking have been noted in the USA, where iodine has been reported in bread in concentrations up to 260 μg/slice—and where, in addition, milk has been described as a major source of iodine. In Tasmania, where the change in bread-making technology was implicated in the 1966 rise in thyrotoxicosis, the most likely cause of the lesser increases in 1964 and 1971 is thought to have been the introduction of iodophors as disinfectants in the dairy industry. Iodophors were introduced in Tasmania in 1963 for the purpose of washing udders and cleaning milking machines and storage vats, and in 1970 the major milk distributors began to use these compounds in their road tankers, bulk-storage vats and bottling plants. This use of iodophors has been shown to result in iodine residues in milk. Iodine concentrations of 113–346 μg/litre have been reported, compared with a maximum of 23 μg/litre in milk from farms not using iodophors. Even the lowest concentration of 113 μg/litre is equivalent to about 60 μg iodine in a pint of milk, and such an increase in an iodine-deficient area like Tasmania represents a substantial change in dietary intake.

In addition to the sudden increases due to these technological innovations, there had been a gradual increase in the iodine content of the Tasmanian diet since the introduction of roll-on roll-off ferries in 1959 had paved the way for improvements in freight services to and from the Australian mainland. This led to an increased consumption in Tasmania of foodstuffs produced on the iodine-sufficient mainland and generally helped to alleviate iodine deficiency except in the most isolated areas, but by itself it could not explain the sudden increase in the incidence of thyrotoxicosis in 1964–5.

The authors suggest that when technological developments resulting in a marked increase in dietary iodine are introduced into previously iodine-deficient areas the risk of iodine-induced thyrotoxicosis is also introduced, and they consider that even iodine-replete areas are not free from such a risk; the incidence of goitre is not restricted to iodine-deficient areas and iodine deficiency is not a prerequisite for iodine-induced thyrotoxicosis. They propose, therefore, that the monitoring of iodine nutrition by measurement of urinary iodine excretion or of thyroid uptake of radio-iodine is desirable in all areas and essential in areas of endemic goitre. Unless specifically suspected, iodine-induced thyrotoxicosis may not be recognized, and therefore clinicians should be alerted to any increase in dietary levels of iodine. The conclusion is drawn that although controlled correction of iodine deficiency is desirable, the unselective addition of iodine to common foodstuffs should be severely restricted.

[It seems that the addition of iodine to the diet presents a hazard to a section of the population in areas of iodine deficiency. The proportion affected could be appreciable since the incidence of goitre in these areas is often quite high. Increases in iodine intake in other areas are likely to present a hazard only to those already suffering from thyroid disease either in a subclinical or overt form.]

AGRICULTURAL CHEMICALS

3027. Pesticide residue levels for German babies

Rappl, A. u. Waiblinger, W. (1975). Zur Kontamination von Muttermilch mit Rückständen chlorierter Kohlenwasserstoffe. *Dt. med. Wschr.* **100**, 228.

Significant residues of *p,p'*-DDT and its metabolite, *p,p'*-DDE, are reported to have been found in 136 of 137 samples of human breast milk collected in Bavarian hospitals between June 1973 and January 1974. DDT concentrations ranged from

0·002 to 0·19 ppm (mean 0·032 ppm), and DDE concentrations from 0·015 to 0·32 ppm (mean 0·065 ppm). With the relatively small amounts of the further metabolite, *p,p'*-DDD, found in 73 of the milk samples, the total concentration of DDT and its metabolites ranged from 0·015 to 0·48 ppm (mean 0·107 ppm). This represented, on average, a fourfold excess and, at worst, a 16-fold excess over the WHO assessment of the acceptable daily intake of DDT for adults.

In addition, γ-BHC (mean concentration 0·012 ppm) was detected in 34 samples, technical BHC (0·069 ppm) in 48, hexachlorobenzene (0·029 ppm) in 112, heptachlor epoxide (0·003 ppm) in 32, and polychlorinated biphenyls (0·10 ppm) in 30. All but three samples, however, were free from residues of aldrin and dieldrin.

PROCESSING AND PACKAGING CONTAMINANTS

3028. Alkane metabolism in rats

Tulliez, J.-E. et Bories, G.-F. (1975). Métabolisme des hydrocarbures paraffiniques et naphténiques chez les animaux supérieurs. I. Rétention des paraffines (normal, cyclo et ramifiées) chez le rat. *Annls Nutr. Aliment.* **29**, 201. II. Accumulation et mobilisation chez le rat. *ibid* **29**, 213.

The presence of hydrocarbons in mammals is well established, but the biological role of these compounds, which are often considered to be inert, is not clear. Normal and branched-chain alkanes and cycloalkanes have been detected in the tissues of many animals, including man. The origin of such alkanes is generally attributed to foodstuffs, particularly vegetables, and in the case of the cycloalkanes to the contamination of food with mineral oils. The amount ingested by man is constantly increasing, owing to the increased use of paraffin oils in the food industry, for therapeutic ends and in low-calorie diets and to the consumption of animal tissues containing accumulated alkanes. In the USA, the intake from food has been estimated at 400 g/head/yr and from mineral oils at an additional 50 g/head/yr.

In the first paper cited above, the authors report the hydrocarbon retention, both apparent (ingested minus excreted) and true (residual, non-metabolized concentration), in rats and the dependency on type of alkane and chain length. The alkanes studied were heptadecane ($C_{17}H_{36}$), eicosane ($C_{20}H_{42}$), heneicosane ($C_{21}H_{44}$), tetracosane ($C_{24}H_{50}$), dotriacontane ($C_{32}H_{66}$), dodecylcyclohexane ($C_{12}H_{25} \cdot C_6H_{11}$), heptadecylcyclohexane ($C_{17}H_{35} \cdot C_6H_{11}$), 2,2,4,4,6,8,8-heptamethylnonane ($[CH_3]_7 \cdot C_9H_{13}$) and 2,6,10,14-tetramethylpentadecane ($[CH_3]_4 \cdot C_{15}H_{28}$).

Each alkane, dissolved in the peanut-oil component of the diet, was fed in a single dose of 15 mg to eight female rats. Preliminary studies of control rats and feed showed the presence of only trace quantities of the alkanes under study. Alkanes were extracted into hexane and analysed by gas chromatography. For 10 days after dosing, urinary and faecal levels of alkane were studied in three of the rats in each group. The eight rats in each group were killed at intervals from day 1 to day 21 and the carcasses were analysed for alkane content.

No urinary excretion was detected; faecal excretion was 2–30% of intake, mostly during days 1–3, but the totals for identically treated animals showed marked variation. Apparent retention was thus high even for the long-chain alkanes.

From these results and from the concentration of unchanged alkanes in the carcass, the authors distinguished two categories of alkanes. Firstly, the branched-chain alkanes, although giving significant apparent retention, were retained only at trace levels in a non-metabolized form. Secondly, variation in retention of the normal alkanes and cycloalkanes was dependent only on the number of carbon atoms. There seemed to be a discontinuity between C_{20} and C_{21}; for alkanes $\leqslant C_{20}$ true retentions 15 days after injection were of the same order (8%), while for those $\geqslant C_{21}$ retention tended to zero.

The residual alkane concentration dropped rapidly on days 1–4 and then levelled out. From these data it was postulated that accumulation might occur upon continuous administration of some alkanes, particularly heptadecane, eicosane and dodecylcyclohexane.

In the second paper, a preliminary study was carried out to locate preferential adsorption sites for eicosane and dodecylcyclohexane administered to rats. Rats were fed with 15 mg of one of these alkanes daily for 7 days and killed on day 10. Most of the alkane detected in the rats was in the fatty tissue. The total residual amounts of eicosane and dodecylcyclohexane were 7·2 and 6·0%, respectively, of the total amount ingested, and compared favourably with the figure of 8% reported after a single dose, thus offering support for the accumulation hypothesis.

In a long-term study, three groups of rats were fed *ad lib.* either a standard diet, the same diet containing 0·1% eicosane or the diet with 0·1% dodecylcyclohexane for 6 months or until a constant level of alkane was observed in the rats. Periodically, one animal from each group was killed and various organs and tissues, in particular the fatty tissue, were analysed for the alkane administered. Three days before the rats were killed, diets containing alkane were replaced by the standard diet.

Alkane accumulated linearly over a long period, and rates of accumulation were identical for both alkanes. Furthermore, the ratio of alkane accumulated in the fatty tissue to the total lipid weight was constant. The accumulated amount of dodecylcyclohexane levelled off after 2 months, but that of eicosane was still increasing when the study period ended at 6 months. The total lipid weights of the rats fed dodecylcyclohexane were 16·5% lower than those of the rats fed eicosane, while the body weights differed by less than 4%.

After 3 and 6 months, respectively, for the rats fed on dodecylcyclohexane and eicosane, administration

of the alkane was stopped and the rats were divided into two further groups. One group was fed a standard diet, the other an energy-reduced diet. In the former group, mobilization of accumulated alkane was slow, only two thirds disappearing in 4 months. In the latter group, mobilization was increased by a third, but the rate was still lower than that of lipid mobilization. As a result, the concentration of alkane in the fatty tissue increased considerably in the rats fed an energy-restricted diet, and estimation of the concentrations of alkanes towards the end of the study period was impossible owing to the presence of insufficient tissue.

3029. Further steps in DEHP metabolism

Albro, P. W. (1975). The metabolism of 2-ethylhexanol in rats. *Xenobiotica* **5**, 625.

Di-(2-ethylhexyl) phthalate (DEHP), commonly known in the industry also as DOP, is one of the most widely used plasticizers, with an annual world production in excess of 300 million tons. DEHP given orally has been shown to be degraded by the contents of the rat caecum or small intestine to a metabolite tentatively identified as mono-(2-ethylhexyl) phthalate (Rowland, *Fd Cosmet. Toxicol*, 1974, **12**, 293). Other metabolic studies have concentrated on the fate of this monoester, but the study cited above was designed to elucidate the metabolic pathway of the simultaneously produced 2-ethylhexanol.

2-Ethyl[1-^{14}C]hexan-1-ol (1 μCi, 8·8 μg) was administered to four male rats by gavage in 0·4 ml cottonseed oil, and two of these rats were given additionally 0·1 ml unlabelled 2-ethylhexan-1-ol. Urinary metabolites were extracted with ether and identified using gas-liquid chromatography and mass spectrometry. The structure of the metabolites was confirmed by esterification and further spectroscopy.

Radioactivity expelled as $^{14}CO_2$ reached peak specific activity in less than 2 hr, while 41% of the total ^{14}C excretion in the urine occurred in the 11–12 hr period after administration, a much lower, steady output being maintained before and after this period. In total, 6–7% of the radioactivity administered was excreted as respiratory $^{14}CO_2$, 8–9% appeared in the faeces and 80–82% was found in the urine, elimination being essentially complete 28 hr after administration.

Metabolites accounting for 78% of the urinary radioactivity were identified, the major metabolite being 2-ethylhexanoic acid. Approximately 8% of the dose of ethylhexanol was excreted as 4- and 2-heptanones in a ratio of 1·27:1. In the rats given only labelled 2-ethylhexanol, the total molar amount of these unlabelled ketones matched the molar amount of $^{14}CO_2$ excreted. It was postulated, therefore, that the ketones arose from partial β-oxidation and subsequent decarboxylation of the 2-ethylhexanoic acid. Other metabolites identified were 2-ethyl-5-hydroxyhexanoic acid, 2-ethyl-5-ketohexanoic acid and 2-ethyl-1,6-hexanedioic acid. Only 3% of the ethylhexanol was excreted unchanged.

The author presents a tentative metabolic pathway for the alcohol. All the excretion products were rationalized in terms of simple oxidation by alcohol and aldehyde dehydrogenases followed by either ω-, $(\omega-1)$- or β-oxidation.

THE CHEMICAL ENVIRONMENT

3030. Arsenic in the air

Blot, W. J. & Fraumeni, J. F., Jr. (1975). Arsenical air pollution and lung cancer. *Lancet* **II**, 142.

Arsenic, a common component of non-ferrous ores, is emitted as an air-borne or solid by-product during processing of the ores and has been incriminated in deaths from cancers of the respiratory tract occurring among workers in metal smelters (*Cited in F.C.T.* 1972, **10**, 100). The high risk of lung cancer in workers exposed to fumes of arsenic trioxide is recognized, but the hazard to residents living near, and especially to windward of, such smelting plants has hitherto not been assessed.

From census data and 1950–1969 mortality records, the authors of the paper cited above have deduced that lung cancer mortality was significantly higher in this period among both men and women living in 36 counties where copper, lead and zinc smelting and refining was carried on than in other US counties where other non-ferrous ores were processed. The mean increase, after correction for demographic factors, was 17% for males and 15% for females. For both sexes the lung-cancer rate was raised in each 5-yr interval of the 20-yr study. In nearly all the copper-, lead- and zinc-mining counties with the highest excesses of lung-cancer mortality, far more people were engaged in smelting and refining than in any other manufacturing industry; mining was the major occupation in two others and the other exceptions were counties with very large populations. Nevertheless, even in counties most heavily involved, workers in the smelting industry accounted for only a small proportion of the total population (less than 1% in half of these counties and less than 3% in all but four). Occupational exposure cannot, therefore, account entirely for the high risk of lung cancer. Instead, air pollution in the neighbourhood of industrial emissions of arsenic is considered to be the most likely explanation for the mortality figures. Sulphur dioxide and other industrial agents may add their quota to the arsenic hazard.

3031. Searching for the basis of chromium carcinogenicity

Schoental, R. (1975). Chromium carcinogenesis, formation of epoxyaldehydes and tanning. *Br. J. Cancer* **32**, 403.

Chromium has been implicated in the relatively high incidence of nasal and lung tumours in industries dealing with the extraction of chromium from its ores, with chromium plating and with the production of chromium pigments (*Cited in F.C.T.* 1976, **14**, 215). However, little success has been achieved in most attempts to induce tumours by treating experimental animals with various chromium preparations, the one exception being the sc injection of calcium chromate suspended in arachis oil. Eighteen out of 24 rats treated in this way developed sarcomas at the site of the sc injections.

The author cited above suggests that this effect of chromate in arachis oil may be due to the formation of carcinogenic aldehydes and epoxyaldehydes as a result of the oxidizing action of the hexavalent chromium on the glycerol and fatty acids produced locally by the enzymatic hydrolysis of arachis oil. Glycidal, an epoxyaldehyde derived from glycerol via acrolein, is known to be carcinogenic to rats and mice when administered by the sc route and 3,4,5-trimethoxycinnamaldehyde is also carcinogenic (*ibid* 1974, **12**, 289).

Epoxyaldehydes derived from oxidized unsaturated oils or quinonoid oxidation products of the polyphenolic constituents of vegetable tannins are thought to be responsible for effecting the cross-linking of free amino groups in collagen, the basic chemical change involved in the transformation of animal skin into leather. Such cross-linking could be brought about in other types of cellular macromolecules, including those of the nuclear chromatin, and one consequence of this might be tumour induction. In this connexion, it is of some interest that certain vegetable tannins induce tumours when injected into rodents, while an increased incidence of nasal tumours reported among workers exposed to leather dust in the shoe industry (*ibid* 1971, **9**, 601) raises the possibility of the involvement of some product of the tanning industry.

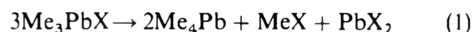
[An attractive hypothesis for armchair discussion—but one based on insecure foundations. While 3,4,5-trimethoxycinnamaldehyde has been shown to induce nasal tumours in rats (*Cited in F.C.T.* 1974, **12**, 289), the argument for the carcinogenicity of calcium chromate and of glycidal rests on the induction of local sarcomas in this species, and for several reasons this route is considered an unreliable index of carcinogenic activity. However, one cannot brush aside the epidemiological evidence implicating chromium as a carcinogen in man, and this whole question is clearly one of considerable importance.]

3032. Methylation of lead by micro-organisms

Schmidt, U. & Huber, F. (1976). Methylation of organolead and lead(II) compounds to $(CH_3)_4Pb$ by microorganisms. *Nature, Lond.* **259**, 157.

Inorganic lead compounds can be methylated by micro-organisms, and alkyllead compounds can be methylated by micro-organisms and/or by disproportionation via the alkyllead sulphide (*Cited in F.C.T.* 1976, **14**, 72) to give tetramethyllead (Me_4Pb). The biological methylation of Pb^{2+} to Me_4Pb has now been confirmed and suggests a route for the biological methylation of trimethyllead acetate (Me_3PbOAc) to Me_4Pb.

In aqueous solution, trimethyllead compounds (Me_3PbX) decompose slowly according to the equation:



$$3Me_3PbX \rightarrow 2Me_4Pb + MeX + PbX_2 \quad (1)$$

The concentration of Me_3PbCl added to water samples seeded with micro-organisms from an aerated aquarium decreased faster, under both aerobic and anaerobic conditions, than was the case in sterile samples, and the proportion of products was no longer in agreement with those predicted by equation (1), less Pb^{2+} but more Me_4Pb being formed. This excluded the possibility that Me_4Pb was produced only by redistribution according to equation (1), and suggested methylation of the Me_3PbCl and/or Pb^{2+} formed in the redistribution.

Incubation of $Pb(OAc)_2$ under the same anaerobic conditions produced Me_4Pb in the atmosphere above the solution, the maximum amount after 7 days being 103 μg Me_4Pb from 100 ml of solution containing Pb^{2+} at 1 μg/ml. Thus besides redistribution according to equation (1), biological methylation of the Pb^{2+} formed in this redistribution must also be considered as a source of Me_4Pb in the experiments with Me_3PbOAc (*ibid* 1976, **14**, 72). The possibility of direct biological methylation of Me_3PbOAc has still to be investigated.

Under conditions of high sulphide concentration, the chemical mechanism would predominate, since the redistribution rate increases with increasing polarizability of the anion X in equation (1), and any Pb^{2+} formed in redistribution would be precipitated as PbS, leaving insufficient Pb^{2+} in solution to allow appreciable microbial methylation to Me_4Pb.

3033. Lead intake from printed paper

Bogden, J. D., Joselow, M. M. & Singh, N. P. (1975). Extraction of lead from printed matter at physiological values of pH. *Archs envir. Hlth* **31**, 442.

Lead contamination has been identified in a wide variety of environmental materials accessible to young children. A recent article on lead exposure and its significance (*Cited in F.C.T.* 1975, **13**, 277) dealt with the intake, by children, of lead from generally recognized sources. The paper cited above provides evidence that the pica-prone child who swallows printed material may also risk absorbing excessive amounts of lead, since a number of samples of coloured printed matter have been found to contain more than 2000 ppm lead, a level 10,000 times greater than the British recommended limit of 0·2 ppm for infant foods.

An *in vitro* study was carried out to investigate whether lead was extracted from printed matter at various pH values, including those likely to be encountered by ingested matter within the digestive tract. Samples of coloured printed matter were divided by colour in order to determine the extent of extraction for the different types. A pair of discs of the printed paper, each 0·25 in. in diameter, was placed in each of ten test tubes containing 2·0 ml distilled water adjusted to pH values in the range 1·0–10·0 and an eleventh pair was placed in a 2·0 ml

sample of human saliva. All were left for a period of 3 hr without agitation. The solutions were then examined for lead content by atomic absorption spectrophotometry. For comparison, a 30 mg paint chip containing 3561 μg lead underwent extraction in an identical series of test solutions.

Lead was extracted from printed matter only at pH values between 1 and 3 and most effectively at pH 1. The greatest amount of lead (202 μg from 140 mg paper at pH 1) was extracted from the yellow samples and the least from the blue (73 μg from 140 mg at pH 1). With these and with the red and green printed papers, the lead extraction rate exceeded 90% at pH 1. No detectable amounts of lead were extractable from white (unprinted) paper at any pH. Lead was readily extracted from the paint chips at pH 1–3 and some (2% of the content) was extracted by solutions in the pH range 4–10, suggesting the presence of a lead compound, such as lead acetate, readily soluble at all of the pH values tested.

The pH of children's saliva ranges from 6·4 to 8·2 and this suggests that chewing the paper without swallowing it would result in little lead extraction. The extraction rate would probably be increased, however, were the child to swallow the material, with or without chewing it, since the normal pH range of children's gastric fluid is 0·9–1·7.

The authors suggest that animal studies should be used to clarify the situation concerning the toxicity of lead in coloured printed matter, as the eating of paper is apparently a relatively common activity among young children.

3034. Lead strikes the foetus

McClain, R. M. & Becker, B. A. (1975). Teratogenicity, fetal toxicity, and placental transfer of lead nitrate in rats. *Toxic. appl. Pharmac.* **31**, 72.

Malformations in the hamster foetus have been reported after iv dosing of the mother with lead (Pb) salts, the primary abnormalities being in the sacral and tail vertebrae (*Cited in F.C.T.* 1969, **7**, 259). It is clear, therefore, that transmission of inorganic Pb across the placenta must occur.

In the study cited above, female rats were given a single iv injection of Pb nitrate in a dose of 25, 35, 50 or 70 mg/kg on one of days 8–17 of gestation. On day 22 the animals were killed and the number and position of live, dead and resorbed foetuses were recorded. An injection between day 10 and 16 produced a high incidence of foetal deaths, the resorption rate being 95–100% following a dose of 50 mg/kg on day 12, 13, 14 or 15. Resorption rates were only 15 and 20%, respectively, following the same treatment on day 8 or 9 and they dropped again to below this level in rats injected on day 17. Thus embryonic and foetal sensitivity to Pb was lowest before day 10 and after day 15. All doses given between days 10 and 17 significantly reduced foetal weight and crown–rump and trans-umbilical distances.

External malformations appeared only after the dose was given on day 8 or 9, with a maximum incidence following treatment on day 9. They were limited to the posterior region of the body and ranged from the absence of a tail to the entire absence of the posterior trunk or the absence of external genitalia, accompanied by imperforate anus. The only other day when malformations were induced was day 16, when Pb produced hydrocephalus and haemorrhage in the central nervous system. Postnatal survival of litters from an extra group of rats given 50 mg Pb/kg on day 9 was poor.

After an injection of ^{210}Pb nitrate on day 17 of gestation, radioactivity was highest in the maternal liver, kidney and spleen. Disappearance of ^{210}Pb from the blood was exponential over 24 hr. Radioactivity was also demonstrated in the placenta, in which its decline paralleled that in maternal blood. At the end of the 24-hr period, the Pb levels in foetus and placenta were 8·1 and 3·1 μg/g, respectively. Infusion of Pb iv at a rate of 0·7 mg/kg/min produced a roughly linear rise in maternal whole-blood and plasma concentrations to 425 and 310 μg/ml, respectively. The placenta clearly exerted some barrier effect, since after infusion for 64 min the whole-foetus concentrations of Pb did not exceed 2·4 μg/g.

3035. Another trace metal study in rats

Schroeder, H. A. & Mitchener, M. (1975). Life-term studies in rats: Effects of aluminum, barium, beryllium, and tungsten. *J. Nutr.* **105**, 421.

We have previously recorded some of the effects attributed to aluminium (*Cited in F.C.T.* 1973, **11**, 139), barium (*ibid* 1974, **12**, 278), beryllium (*ibid* 1974, **12**, 760) and tungsten (*ibid* 1968, **6**, 539), four elements that have now been included in a continuing series of experiments in rats and mice on the effects of low doses of trace metals in the diet (*ibid* 1976, **14**, 217).

Groups of male and female rats were given, from weaning to natural death, drinking-water containing one of these metals in a concentration of 5 ppm in the form of a soluble salt (potassium aluminium sulphate, barium acetate, beryllium sulphate or sodium tungstate). In addition to the test metal, all samples of drinking-water contained constant levels of trivalent chromium, zinc, copper, manganese, cobalt and molybdenum. The rats were weighed weekly at first, then at monthly intervals for 1 yr and subsequently at 3-monthly intervals. When the rats died, they were autopsied and heart, lung, kidney, liver and spleen tissues and any tumours were examined microscopically. Serum glucose and fasting levels of blood cholesterol and uric acid were measured, and urinary protein, pH and glucose were checked. Tissue levels of the trace metals were not determined. When the rats were 20 months old, an epidemic of pneumonia killed 139 males and 113 females, the deaths being fairly evenly distributed between the control and test groups, each of which had initially consisted of 52 males and 52 females, apart from the tungsten group which contained 37 males and 35 females. This epidemic did not appear to affect the overall findings, which were largely negative.

Among the rats fed aluminium, the males—but not the females—showed a slightly increased incidence of tumours and were slightly heavier after the first 12 months of dosing; no other significant effects were

noted. Barium caused few significant effects, the females only being slightly overweight in the latter part of the study and having a slightly shorter lifespan, while the males showed some proteinuria. Beryllium similarly caused few adverse effects, the main changes being growth depression in the males between 2 and 6 months and, in females, some glycosuria and a slight reduction in longevity. Tungsten appeared to be the most toxic of the four elements tested. Both males and females had slightly enhanced body weights after about 1 yr, the lifespan was significantly decreased in the males and a similar trend was noted in the females. Overall, however, the effects of these four elements were minimal at the low dose given.

3036. Tissue penetration by ingested mineral fibres?

Gross, P., Harley, R. A., Swinburne, L. M., Davis, J. M. G. & Greene, W. B. (1974). Ingested mineral fibers. Do they penetrate tissue or cause cancer? *Archs envir. Hlth* **29**, 341.

It is known that mesothelioma of the pleura can be induced readily in the rat by direct intrapleural application of asbestos, and this has offered a means for investigating the carcinogenic mechanisms involved (*Cited in F.C.T.* 1974, **12**, 591). In addition to inhalation of airborne asbestos, human exposure can occur through consumption of foods and beverages containing asbestos fibres. The results of one animal experiment (Pontefract & Cunningham, *Nature, Lond.* 1973, **243**, 352) showed that asbestos fibres could migrate through the gastro-intestinal wall into the blood stream and accumulate in various tissues, but the FDA considered that, overall, the evidence was inconclusive and therefore initiated a joint FDA/Environmental Protection Agency feeding study on asbestos. If migration of asbestos fibres through the gastro-intestinal wall does occur, this could provide an alternative absorption route for fibres that are cleared from the lungs and swallowed. In contradiction to the results of Pontefract & Cunningham (*loc. cit.*), however, the study cited above indicates that mineral fibres do not penetrate into the tissues of rats.

This joint paper presents the pooled results of investigations into asbestos ingestion carried out in three different laboratories. The treatments involved were (a) chrysotile feeding (5% by weight of the diet), (b) administration of fibrous minerals by gavage (1 ml of a suspension containing 400 mg amosite or taconite dust), (c) feeding of oleomargarine with a 10% amosite or 20% taconite content, (d) feeding of butter containing various concentrations of crocidolite and (e) long-term *ad lib.* feeding of asbestos in butter (chrysotile or crocidolite in a concentration of 5 mg/g). Examination for fibres was carried out both by electron microscopy of tissue digests and by light microscopy of tissue sections.

There was no evidence of tumour induction or of the promotion of any other kind of lesion after 21 months of intimate contact of rat gastro-intestinal mucosa with very high concentrations of chrysotile asbestos, nor was there any indication of penetration or transmigration of the gut wall. Administration by gavage and voluntary consumption in oleomargarine similarly provided no evidence for the transmigration of fibres. Likewise long-term feeding of chrysotile and crocidolite for up to 1·5 yr resulted in no tumour production related to asbestos intake nor was there any evidence of tissue penetration.

It is thought that the gastro-intestinal mucosa provides a far more formidable barrier than the pulmonary alveolar membrane, in which trans-migration of inhaled dust is readily demonstrable by the presence of enlarged, discoloured, mineralized tracheobronchial lymph nodes. No similar involvement of the mesenteric lymph nodes is seen in miners, although during their lifetime they ingest approximately 100 times as much dust as becomes stored in their lungs and satellite lymph nodes, in addition to the large amount of insoluble particulates to be found in the daily food intake. Moreover, no evidence of penetration or storage of ingested foreign material has been found in the intestinal wall of such miners.

3037. The dimensions of respirable fibreglass

Dement, J. M. (1975). Environmental aspects of fibrous glass production and utilization. *Envir. Res.* **9**, 295.

Studies seem to suggest that the dimensions of fibrous glass particles are a key factor in fibre carcinogenesis and other pathogenic responses. It was reported that exposure to fibres of diameter below 3·5 μm at one pilot plant may have been associated with the development of respiratory cancer in four workers (*Cited in New Scientist* 3 April 1975, **66**, 19) although a more recent investigation, which unfortunately did not establish the size of the fibres, showed no evidence of increased cancer induction as a result of exposure to fibreglass (*Cited in F.C.T.* 1976, **14**, 158). The aim of the study cited above was to determine fibreglass levels in various types of industrial operation, paying special attention to exposure to small-diameter fibres. Large-diameter fibres are used to make such products as pipe insulation; small-diameter fibres are used in the manufacture of high-efficiency filter paper and high-temperature insulation.

Low-level exposures were recorded in large-diameter fibreglass insulation operations where the highest mean fibre concentration was 0·20 fibres/ml and the highest mean total airborne dust concentration was 4·4 mg/m^3. The smallest median airborne fibre diameter recorded in these facilities was 1·1 μm and the smallest length 19 μm. Of these airborne fibres, the proportion less than 5 μm in length ranged only from < 1 to 7%. In the small-diameter fibre operations examined, the mean airborne fibre concentrations ranged from 1·0 to 21·9 fibres/ml. None of the facilities sampled had a mean concentration of total airborne dust in excess of 1·0 mg/m^3. Under these conditions, 40–85% of all airborne fibres were less than 0·5 μm in diameter and 5–45% were less than

5 μm in length. In a similar investigation of reinforced-plastics operations, the median airborne fibre diameter was approximately 5 μm and the median length was 35 μm. Virtually no fibres shorter than 5 μm were observed in these operations.

In assessing possible respiratory effects, it is necessary to determine the proportion of airborne fibres actually reaching the alveolar regions. A certain amount of controversy exists as to fibre respirability, and in particular as to the upper limit of fibre length in this connexion. This report suggests that a potentionally respirable fibre may be defined as being less than 3·5 μm in diameter and less than 50 μm in length. From these investigations it appears that most respirable fibres occur in those operations manufacturing and using small-diameter fibres. Animal studies have indicated that exposures to respirable glass fibres should be kept at an absolute minimum.

3038. Acetaldehyde overexposure

Kruysse, A., Feron, V. J. & Til, H. P. (1975). Repeated exposure to acetaldehyde vapor. Studies in Syrian golden hamsters. *Archs envir. Hlth* **30**, 449.

Acetaldehyde is known to be an important toxic component of the gas phase of tobacco smoke, and studies have shown that the compound is retained in the upper respiratory tract after inhalation (*Cited in F.C.T.* 1973, **11**, 922). The subject of acetaldehyde and its toxic effects has recently been reviewed (*ibid* 1975, **13**, 668) and the purpose of the study cited above was to obtain background toxicological data on acetaldehyde to aid in the assessment of the importance of aldehydes from cigarette smoke in the genesis of pulmonary cancer.

Four groups each of ten male and ten female hamsters were repeatedly exposed to acetaldehyde vapour at concentrations of 0, 390, 1340 or 4560 ppm for 6 hr/day on 5 days/wk over a 90-day period. The highest level caused growth retardation, ocular and nasal irritation, increased numbers of erythrocytes, increased weights of heart and kidneys and severe histopathological changes in the respiratory tract (mainly necrosis, inflammatory changes and hyper- and metaplasia of the epithelium). At 1340 ppm, changes that could be attributed to the test compound were only slight. These included increased kidney weights in males and slight hyper- and metaplastic changes in the tracheal epithelium. A concentration of 390 ppm was considered to be the no-toxic-effect level.

This latter level fits well with the 100 ppm TLV (threshold limit value) for acetaldehyde specified by the American Conference of Governmental Industrial Hygienists (TLVs: Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Environment with Intended Changes for 1975; ACGIH, P.O. Box 1937, Cincinnati, Ohio 45201, USA). However, the lower of the two concentrations of acetaldehyde that induced histopathological changes in the respiratory tract of hamsters (1340 ppm) was within the range of acetaldehyde concentrations present in cigarette smoke (1100–2000 ppm).

3039. Analgesics and the diet

McLean, A. E. M. & Day, P. A. (1975). The effect of diet on the toxicity of paracetamol and the safety of paracetamol–methionine mixtures. *Biochem. Pharmac.* **24**, 37.

Pantuck, E. J., Hsiao, K.-C., Kuntzman, R. & Conney, A. H. (1975). Intestinal metabolism of phenacetin in the rat: Effect of charcoal-broiled beef and rat chow. *Science, N.Y.* **187**, 744.

It has been suggested (Mitchell *et al. J. Pharmac. exp. Ther.* 1973, **187**, 185 & 211) that the action of the cytochrome *P*-450 system of the liver on paracetamol may produce an active metabolite which causes cell injury. The authors of the first paper cited above argue that low-protein diets reduce liver-glutathione (GSH) levels and cytochrome *P*-450 levels and may therefore affect the animal's sensitivity to the toxic effects of paracetamol. Rats fed a low-protein diet showed a decrease in hepatic GSH and *P*-450 and became more sensitive to the hepatic toxicity of paracetamol, a sensitivity that was further increased when they were pretreated with phenobarbitone to raise the hepatic *P*-450 level. Rats fed a 25% yeast diet, which reduced GSH content while leaving *P*-450 levels unaltered, were most susceptible of all. In all the dietary groups, great variability in hepatic damage was observed. Rats given methionine orally with the analgesic at a level equivalent to 10% of the paracetamol dose were relatively free from hepatic injury. It appears that certain nutritional factors may affect the metabolic pathway open to paracetamol by promoting either sulphation or glucuronidation to safer metabolites or oxidation by the *P*-450 system to toxic ones. When toxic metabolites are formed they may react either with cellular macromolecules or with GSH or cysteine, so at this point yet another safety factor may be offered by an adequate diet.

The second paper cited above describes the feeding of rats with a chow diet in place of a semi-synthetic casein/starch/vegetable-oil diet for 7 days. Segments of small intestine taken from these animals showed a 200% increase in ability to metabolize phenacetin *in vitro*. Segments from other rats fed charcoal-broiled ground beef (3 parts) plus the semi-synthetic diet (1 part) showed an increase of 1100% in phenacetin-metabolizing capacity. In contrast, the increase in phenacetin metabolism produced by feeding raw or foil-cooked beef was only about 100 and 50%, respectively. The authors point out that charcoal-broiled beef contains polycyclic aromatic hydrocarbons, which are known to stimulate metabolic processes in the rat liver and placenta. The effect of such factors in the diet may provide a partial explanation for the wide variations in drug metabolism observed in man.

3040. Hypersensitivity to chlorocresol

Hancock, B. W. & Naysmith, A. (1975). Hypersensitivity to chlorocresol-preserved heparin. *Br. med. J.* **3**, 746.

Chlorocresol has been used as a preservative in

pharmaceuticals for several years and contact sensitization is known to be associated with the cresols (Fisher, *Contact Dermatitis*. 2nd ed., p. 370, Lea & Febiger, Philadelphia, 1972). This paper reports on two recently observed cases of generalized reaction and seven of local reaction to mucous heparin containing 0·15% chlorocresol. Allergic reactions to heparin are rare, and in all but one of these cases hypersensitivity to the chlorocresol was implicated. The first of the two cases of generalized reaction, a woman with deep-vein thrombosis and a pulmonary embolism, collapsed with pallor, sweating, hypotension and tachycardia after a second iv dose of 10,000 units chlorocresol-preserved mucous heparin. The second, a man with identical ailments, developed nasal congestion, profuse sweating and a generalized urticarial rash after being given 10,000 units of heparin iv. Localized reactions consisting of indurated erythema were observed in seven established or suspected cases of myocardial infarction receiving sc heparin (10,000 units) twice daily.

In the patients given an intradermal skin test with chlorocresol-preserved heparin, an immediate response was observed, whereas in only one of the six cases tested was there a reaction to preservative-free heparin.

The authors suggest that in cases of apparent heparin sensitivity, it may be possible to continue treatment without adverse reaction after challenging with chlorocresol-free heparin.

3041. Ocular effects of a fluorocarbon

Brubaker, S., Peyman, G. A. & Vygantas, C. (1974). Toxicity of octafluorocyclobutane after intracameral injection. *Archs Ophthal.* **92**, 324.

The use of fluorocarbons as aerosol propellants and refrigerants is well known, but there are several more unusual uses for these materials. The paper cited above describes a study of the intraocular effects of octafluorocyclobutane (C_4F_8), which is being considered as a possible replacement for sulphur hexafluoride (SF_6) in retinal-detachment surgery. The surgical technique in question involves injection of the gas into the eye to tamponade the retina to the pigment epithelium.

Anaesthetized rabbits were divided into three test groups and one control group. The latter was given sham injections, while the test groups were injected intraocularly with either 0·15 ml air, 0·15 ml SF_6 or 0·15 ml C_4F_8, in order to displace most of the aqueous humour. In the light of the results of the initial work, further injections were carried out with volumes of 0·03–0·12 ml C_4F_8 or SF_6, or 0·15 ml of either a 40:60 SF_6–air mixture or a 40:60 C_4F_8–air mixture. The size of the gas bubbles was observed daily and each eye was examined with a slit-lamp and by tonometry, fundoscopy and serial photography. Histological studies were carried out 2, 4 and 30 days after injection. In some cases the cornea was sectioned and stained for electron microscopy.

Only mild changes resulted from injection of air. Mild ciliary hyperaemia and corneal haze at the periphery of the gas bubble was sometimes apparent, mild iritis was common and opacification of the anterior lens capsule also developed in some cases, but no significant long-term changes were seen. However, both SF_6 and C_4F_8 caused acute reactions, notably marked hyperaemia, cloudy oedematous corneas and severe iritis. Cataracts also formed, and a rise in ocular pressure frequently led to atrophy of the iris and pupillary dilation as late complications. In the tests on graded volumes of SF_6 and C_4F_8, it was found that reactions occurred with injections of 0·10 ml or more. With volumes below this, reaction was limited in some 50% of the eyes to ciliary hyperaemia, corneal haze at the periphery of the bubble and moderate iritis. The mixtures of SF_6 or C_4F_8 with air caused reactions similar to those evoked by small volumes (less than 0·10 ml) of the pure test gases. Some changes involving the layering of the endothelial cells over the gas bubble were seen by electron microscopy following injection of C_4F_8 or SF_6.

C_4F_8 consistently lasted longer than SF_6, but both gases behaved in a similar manner and expanded after injection into the eye. Corneal opacification was a reversible phenomenon but opacification of the anterior lens capsule was permanent. Enlargement of the cornea and atrophy of the iris were consistent findings at high intraocular pressure. However, this pressure gradually returned to normal after 2–4 wk and hyperaemia of the conjunctiva disappeared. Since C_4F_8 toxicity was comparable to but no greater than that of SF_6, it was concluded that this fluorocarbon might be a suitable material for vitreous replacement, although further studies in primates would be required to confirm its safety in such use.

3042. Carcinogenicity of nitrosation products of environmental chemicals

Wogan, G. N., Paglialunga, S., Archer, M. C. & Tannenbaum, S. R. (1975). Carcinogenicity of nitrosation products of ephedrine, sarcosine, folic acid, and creatinine. *Cancer Res.* **35**, 1981.

N-Nitroso compounds, as a class, are of great biological interest because they include some of the most potent carcinogens known. Carcinogenic *N*-nitroso compounds can be produced as a result not only of environmental interaction of nitrite with suitable substrates but also of nitrosation effected *in vivo* by nitrite ingested as such or derived from dietary nitrate. Because of the latter possibility, attention has been directed to the carcinogenic activity of the nitrosation products of ephedrine, folic acid, sarcosine and creatinine, since all these compounds are readily nitrosatable and the degree of human exposure to them is high. Ephedrine is a commonly used drug and the other three occur widely in edible products.

In a preliminary study of the nitroso derivatives of these four compounds, the LD_{50} (mg/kg) values in day-old mice of the C57BL/6J × C3HeB/FeJF_1 strain were found to be 184 for *N*-nitrososarcosine (NS), 392 for *N*-nitrosoephedrine (NE), 327 for N^{10}-nitrosofolic acid (NF) and 529 for creatinine-5-oxime (CRO). The figure for diethylnitrosamine (DEN), included for purposes of comparison, was

42 mg/kg. For the carcinogenicity study, all five compounds were suspended in trioctanoin and administered to groups of 44–51 newborn mice in a series of ip injections, each in a volume of 5 ml/kg. DEN was given in four doses, each of 3 mg/kg, on days 1, 4, 10 and 16 after birth. The other compounds were given on days 1, 4 and 7, the doses of NS, NE, NF and CRO given being 75, 200, 125 and 300 mg/kg, respectively. DEN induced hepatocellular carcinoma in 11/12 males and 12/13 females, and one cholangiocarcinoma and one pulmonary adenocarcinoma were also observed in the female group. NS induced hepatocellular carcinoma in 8/12 males and liver-cell hyperplasia in 3/12 males and 3/17 females. NE induced only hepatocellular carcinoma, which occurred in all the treated males and 13/15 females. NF induced hepatocellular carcinoma only in one male of the 14 males and 15 females in the group, but lung adenocarcinoma was found in three of the males and one female and a neurofibrosarcoma was present in one male. Similarly, CRO induced only one hepatocellular carcinoma in ten treated animals, but three other males in this group had liver-cell hyperplasia. No lesions were found in the CRO-treated females. All these incidence figures refer to mice surviving for 50–85 wk after dosing. In controls treated with 5 ml trioctanoin/kg, 2/14 males had liver-cell hyperplasia while one female out of 12 had a hepatocellular carcinoma.

NE induced the highest incidence of tumours, followed by NS, both compounds being regarded as carcinogenic. NF gave an equivocal result, suggesting carcinogenic activity but requiring further investigation. CRO produced no evidence of any carcinogenic effect.

3043. Fundamental percutaneous absorption studies

Roberts, M. S. & Anderson, R. A. (1975). The percutaneous absorption of phenolic compounds: the effect of vehicles on the penetration of phenol. *J. Pharm. Pharmac.* **27**, 599.

Vehicle or solvent effects are of importance in the clinical assessment of any topically applied agent. This study illustrates rather elegantly the interactions that occur between a model compound (phenol), various vehicles and a biological membrane (excised rat skin), by comparative experiments using also an inert polyethylene film.

Theoretically it was shown that for a series of solvents there would be a constant ratio of solute flow rates through two membranes as long as no solvent–membrane interaction occurred. The flow rates of phenol through excised rat skin were consistently double those through the polyethylene film when arachis oil, light liquid paraffin or glycerol was used as the vehicle, suggesting that these non-polar solvents could be considered as reference materials that did not interact with the biological membrane. The use of water, dimethylsulphoxide (DMSO), dimethylformamide (DMF) or ethanol as the vehicle produced higher rat skin to polyethylene ratios for solute flow, and it was inferred that there must have been significant interaction between the skin and these polar solvents. Although these flow ratios were much higher than those obtained with the reference solvents (e.g. 18 with DMSO), the actual flow rates were usually very low. The interactions that occurred between the vehicle and the skin were said to decrease the diffusional resistance of the stratum corneum, although alteration in the polarity or the thickness of the stratum corneum may also have occurred.

Interaction of solute and vehicle may affect the availability of the solute. Both the light liquid paraffin/vehicle partition coefficient and the permeability coefficient of phenol through polyethylene varied with the vehicle in a similar manner. Either of these parameters may have reflected the thermodynamic activity of phenol in the vehicle. The permeation of phenol in aqueous solution through the membranes decreased on inclusion of glycerol in the system, and similar reductions occurred on addition of DMSO, DMF or ethanol. In all cases, the miscible solvent was thought to decrease the activity of the solute by increasing its potential maximum solubility.

Skin–solute interactions were also demonstrated in this study. Skin damage was evaluated from the degree of penetration of a non-damaging concentration of phenol before and after treatment of the skin with a damaging concentration of the solute. The penetration ratio increased when the skin had been damaged.

Although this paper deals specifically with the transport of phenol across membranes, the experiments described are of wider significance. With the aid of a biologically inert film for comparison, the various interactions between a drug or toxin and the skin of an animal can be differentiated and more fully understood.

NATURAL PRODUCTS

3044. A new fungal food assessed

Volesky, B., Zajic, J. E. & Carroll, K. K. (1975). Feeding studies in rats with high protein fungus grown on natural gas. *J. Nutr.* **105**, 311.

Guidelines for testing single-cell protein intended for animal feeding have been outlined (Proposed Guidelines for Testing of Single Cell Proteins Destined as Major Protein Source for Animal Feed; IUPAC Technical Reports—no. 12; IUPAC, Oxford,

1974; pp. 26). This paper is concerned with a fungal culture of single-cell protein (SCP) produced by a species of Graphium capable of utilizing a gaseous hydrocarbon substrate, such as natural gas. The investigations comprised a preliminary short-term (7-day) toxicity study and a longer (5-month) feeding test, in which the rats were observed for a further 10 months while maintained on a standard diet.

The composition of the feed biomass was protein 47%, lipids 10%, carbohydrate and fibre 30–33% and ash 9–11%. Rats fed SCP at a level of 40% of the diet for 7 days gained weight, whereas animals fed exclusively on SCP showed a weight loss, which was probably due to a decrease in food intake. No behavioural changes were recorded and no pathological signs were detected at autopsy. In the rats fed 20 or 40% SCP for 5 months, body weights were only 84 and 73%, respectively, of the control value. Some changes in the white-cell counts were apparent after 70 and 100 days on the test diets, but the red-cell counts did not differ significantly from those of the controls. Half of the animals in each group were killed after 100 days. No notable pathological changes were observed in these rats, although the relative weights of some organs were increased, a finding that must be viewed in the light of the reduced body weights of these animals. The remaining rats in each group were transferred to standard rations after 5 months on the test diets and were killed and autopsied after a 10-month observation period. By this time, there was little difference in the body weights of the test and control animals. The animals showed no pathological abnormalities apart from a single cortical adenoma in the kidney of one rat fed a diet containing 20% SCP and 10% casein.

3045. Protective properties of plant fibres

Ershoff, B. H. & Marshall, W. E. (1975). Protective effects of dietary fiber in rats fed toxic doses of sodium cyclamate and polyoxyethylene sorbitan monostearate (Tween 60). *J. Fd Sci.* **40**, 357.

It has been shown that the toxic manifestations induced by large doses of certain compounds can be counteracted by the presence of various materials containing plant fibres. Work on the reduction in the toxic effects of amaranth in the presence of fibre (*Cited in F.C.T.* 1975, **13**, 581) suggested that hemicelluloses and/or other plant ingredients played the major protective role. The study cited above examines the comparative efficacy of some plant fibres and fibre-containing materials in reducing the toxic effects of sodium cyclamate and polyoxyethylene sorbitan monostearate.

The addition of sodium cyclamate at a level of 5% to a purified vitamin-supplemented low-fibre diet containing 66% sucrose, 24% casein, 5% salt mixture and 5% cottonseed oil resulted in a retardation of weight gain, lack of grooming, alopecia and extensive diarrhoea, leading in many cases to death within the 14-day experimental period. Rats fed 15% polyoxyethylene sorbitan monostearate in the basal purified low-fibre diet showed similar reactions. These effects were largely counteracted by various plant fibre-containing materials added to the diet at a level of 10%. Blond psyllium seed powder provided the most protection and carrot-root powder, alfalfa meal and cabbage powder were also potent sources of the active factor(s). Moderate protection was afforded by carrageenan and gum tragacanth. The efficacy of some of the substances was found, however, to differ for the two toxic agents. Thus pectin N.F. and a carboxymethylcellulose, sodium CMC 7HF, were more active in counteracting cyclamate toxicity, whereas cellulose and rice straw proved to be more active against polyoxyethylene sorbitan monostearate.

These results suggest that the protective effects of dietary fibres are probably due to their physicochemical properties and their effect on the physical state of the substrate in the gut rather than to their content of known nutrient factors. Further work on such properties as swelling power, viscosity, gelling, ion-exchange capacity and the ability to bind toxins is needed to elucidate the situation.

3046. Tea-drinkers' anaemia

Disler, P. B., Lynch, S. R., Charlton, R. W., Torrance, J. D., Bothwell, T. H., Walker, R. B. & Mayet, F. (1975). The effect of tea on iron absorption. *Gut* **16**, 193.

It seems that absorption of dietary iron (Fe) is a hazardous business, which can be influenced to an increasingly evident degree by other constituents of the diet. It was reported recently that peptic digestion of liver converts a higher proportion of the Fe to absorbable low-molecular-weight complexes when the liver has been cooked than when it is raw (*Cited in F.C.T.* 1975, **13**, 482) and it now seems that, in this context, the cooking of meat may be an even more desirable custom for tea-drinkers than for others.

The absorption of Fe from ferric chloride ($FeCl_3$) solution, from a solution of ferrous sulphate ($FeSO_4$) containing ascorbic acid, from rabbit haemoglobin or haem in tomato juice or gravy, from Fe-enriched bread, and from a meal of rice with potato and onion soup was studied in Indian women who drank either plain tea or tea containing milk with the test meals. Controls drank warm tap-water instead of tea. The drinking of milkless tea inhibited Fe absorption from the inorganic compounds, and tea with milk reduced absorption from $FeSO_4$. Tea also inhibited the absorption of Fe from enriched bread and from soups carrying an extrinsic label in the form of added $^{59}FeSO_4$ or $^{55}FeSO_4$. Although Fe absorption from uncooked haemoglobin was inhibited by tea-drinking, its absorption from cooked haemoglobin was not. Following administration of $FeSO_4$ with ascorbic acid, iron absorption rates were correlated with serum ferritin concentrations. In a third of the subjects, the serum ferritin concentration was below the normal value of 10 μg/litre.

Iron nutrition is generally satisfactory in communities where cooked meat is an important part of the diet, but iron deficiency is common wherever the staple diet is predominantly of vegetable origin. Tea-drinking is an obvious factor to look for in a community that is anaemic.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3047. A clean slate for zinc pyrithione shampoos

Nolen, G. A., Patrick, L. F. & Dierckman, T. A. (1975). A percutaneous teratology study of zinc pyrithione in rabbits. *Toxic. appl. Pharmac.* **31**, 430.

Previous work on the possible toxicity of zinc pyrithione (ZPT) used at a level of 2% in a shampoo formulation for dandruff has demonstrated that the amounts that could be absorbed under normal conditions of usage present no hazard (*Cited in F.C.T.* 1966, **4**, 554). Now the possible teratological effects of ZPT have been evaluated.

A clipped area (200 cm^2) on the backs of pregnant rabbits was treated with a 2% ZPT cream shampoo in a quantity of 1 or 2·5 g/kg/day (equivalent to 20 or 50 mg ZPT/kg/day) from day 7 to day 18 of gestation. Each application was left for 2 hr and then rinsed off. Two control groups received, respectively, no treatment and treatment with shampoo containing no ZPT. The does were killed on day 28, the numbers of resorption sites and corpora lutea were recorded and the foetuses were examined. Apart from mild primary irritation, which developed at the site of application after 3 days of treatment, no signs of toxicity were noted. There were no significant differences between treated and control animals in any of the reproductive parameters studied or in the incidence of skeletal or soft-tissue defects in the foetuses.

Assuming an in-use human exposure to 10 g shampoo during each hair-washing session, the experimental application of 20–50 mg ZPT/kg/day represents an exposure some 6–15 times greater, and the safety margin is further increased by the exaggerated time of contact used in the experiment. In all, the experimental exposure was calculated to be more than 100 times as great as the expected in-use exposure.

TOXICOLOGY

3048. Contact dermatitis epidemiology

Brun, R. (1975). Epidemiology of contact dermatitis in Geneva (1000 cases). *Contact Dermatitis* **1**, 214.

The 'apparent allergenicity' index has been defined as the frequency with which eczematogenic substances are the cause of contact dermatitis. For an individual substance, the index is most useful for a given population and for a set period of time, although it may well be modified by such factors as changes in fashion, changes in people's habits and the establishment of a new industry. In an attempt to determine a value for the index, the epidemiology of contact dermatitis was investigated in 1000 cases in Geneva and the results were compared with similar European and American studies (Fregert *et al. Trans. St. John's Hosp. Derm. Soc.* 1969, **55**, 17; Rudner *et al. Archs Derm.* 1973, **108**, 537).

Each contact-dermatitis patient was tested routinely with a standard series of substances, namely nickel sulphate, potassium bichromate, phenylmercury borate, *p*-phenylenediamine, formaldehyde, turpentine, Peruvian balsam, Araldite D (resin), Araldite hard, butyl catechol, rubber, methyl- and propylparabens and lauryl gallate, along with other substances. Patches were left on the patient's back for 24–48 hr and after their removal the reactions were read at intervals of 30 min and 1 and 2 days (Beiersdorf tests).

Statistically the allergens could be split into four groups. In the first were those substances responsible for approximately 50% of the cases of contact dermatitis (turpentine, nickel, hexavalent chromium and organomercury compounds), in the second were formaldehyde, rubber and *p*-phenylenediamine, the third contained several known allergens, notably preservative agents, while the fourth was a group of at least 48 other substances, including medical creams, natural products and colouring materials. Age and sex distributions were found to be normal except in the cases of nickel allergy (more common in women) and chromate sensitivity (more common in men).

Comparison with other studies showed the same seven allergens to be responsible for the majority of cases of contact dermatitis in Europe and in the USA. The slight variation between different populations could have been due to local influences. It is suggested by the authors that, as in the industrial world, information should be given to the general public in order to reduce the incidence of contact dermatitis.

PATHOLOGY

3049. Is zinc polycarboxylate cement tumorigenic?

Main, J. H. P., Mock, D., Beagrie, G. S. & Smith, D. C. (1975). Investigation of possible oncogenic action of zinc polycarboxylate cement by implantation in mice and hamsters. *J. biomed Mater. Res.* **9**, 69.

Powdered zinc polycarboxylate with a maximum particle size of 45 μm was sterilized by treatment with ethylene oxide and then implanted sc in young hamsters and mice for 15 and 12 months, respectively, after which the surviving animals were killed and autopsied. Implanted material was still present in one of the 42 surviving mice and in 16 of the 43 hamsters.

Benign adenomas of the gut, thyroid, sebaceous glands and ovary were found in three treated mice and in one sham-operated control. Benign adrenal adenomas occurred in three implanted hamsters, and one control hamster developed a malignant rhabdomyosarcoma of the hind limb. In one experimental hamster, leiomyosarcoma developed adjacent to a zinc polycarboxylate implant. Persistent implants were encapsulated with collagenous fibrous tissue, and most were surrounded by a few chronic inflammatory cells, macrophages and an occasional giant cell of the foreign-body type.

The results indicate that mice are more efficient than hamsters in the active removal of particles of the cement. The one sarcoma in a hamster, representing an incidence of one in 109, was probably related to the implant, but its significance was not clear. The tumorigenic potential of zinc polycarboxylate is evidently low, and may be expected to depend upon the form of the implant, its size and the test species used.

3050. Reactions to sutures in human tissues

Postlethwait, R. W., Willigan, D. A. & Ulin, A. W. (1975). Human tissue reaction to sutures. *Ann. Surg.* **181**, 144.

A systematic study of the reaction of human tissues to different types of suture material was made by examining a small block of tissue, including part of the suture, removed at a second operation or at autopsy. Most of the samples were taken from muscle, fascia or skin. Catgut was examined at intervals up to 11 yr after the original implantation, silk up to 23, cotton up to 7, wire up to 33, nylon up to 10 and Dacron 11 and Dacron coated with Teflon up to 6.

The reaction to catgut was mainly histiocytic and depended on the stage of absorption. Two catgut sutures, one removed after 8 yr and another removed after 11 yr, were encapsulated and showed no evidence of absorption. Most silk sutures remained compact and surrounded by a fibrous capsule of varying thickness. A thin histiocyte layer adjacent to the suture and variable numbers and distributions of giant cells and lymphocytes were seen; the more cellular reactions involved prominent capillaries. A second type of reaction to silk showed invasion of the interstices of the suture by fibroblasts and histiocytes, producing in rare instances a reaction resembling a granuloma. Cotton sutures produced a similar but generally more cellular reaction. The tissue reaction to wire was considerably less that that to silk, and was characterized by fibrous tissue lined with monocytic cells. Nylon provoked least reaction of all, comprising a narrow compact fibrous-tissue zone with occasional histiocytes and rare giant cells. The reaction to Dacron was similar to, but less than, that provoked by silk. Particles of Teflon shed from coated Dacron sutures tended to provoke their own separate histiocytic and giant-cell reaction in the tissues.

These reactions were similar to those already demonstrated in experimental animals, but in general it seems that they were somewhat less intense in the human than in the animal studies.

3051. Leucocyte response to implanted starch

Rigdon, R. H. (1975). Starch and chemotaxis. A histological study in the mouse. *Exp. Path.* **10**, 91.

It has been observed that foreign materials, particularly plastics, implanted in the subcutaneous tissues of mice, rats and rabbits attract polymorphonuclear leucocytes, and the suggestion has been made that this migration is an electrochemical phenomenon, negatively charged leucocytes being attracted to the positively charged plastics surface. This process was given the name 'chemotaxis' by Leber nearly a century ago (*Fortschr. Med., Berlin* 1888, **6**, 460). In the present study, starch granules were implanted sc in mice to gain additional data relevant to the hypothesis that inflammation is an electrochemical phenomenon.

Granules of arrowroot powder were injected sc into 108 mice, and the effects were examined in groups of animals 30, 60 and 90 min, 2, 3, 4, 6 and 24 hr and 5, 45 and 61 days after treatment. The area of starch at the injection site varied from 8 to 15 mm in diameter. Tissue sections were removed and stained, generally with haematoxylin and eosin but in selected cases by the Giemsa technique.

Oedema, observed around the starch 2 hr after treatment, increased in severity for the first 24 hr. No oedema was apparent by day 5. Histologically, some leucocytes were seen around the starch granules in two of the five animals examined at 30 min, in four at 60 min and in all five at 90 min. Up to 24 hr the numbers of leucocytes increased, and by that time there was a wide zone of cells infiltrating the periphery of the starch deposit. By day 5 the number of leucocytes had decreased and some mononuclear cells and fibroblasts were present. By day 45 a narrow band of fibrous tissue surrounded and infiltrated the starch deposit. The increase and decrease in oedema appear to parallel the increase and decrease in numbers of leucocytes, and it is suggested that both may be induced by the same mechanism.

CANCER RESEARCH

3052. A rat model for detecting bladder carcinogens

Hicks, R. M., Wakefield, J. St. J. & Chowaniec, J. (1975). Evaluation of a new model to detect bladder carcinogens or co-carcinogens; results obtained with saccharin, cyclamate and cyclophosphamide. *Chemico-Biol. Interactions* **11**, 225.

The artificial sweeteners, cyclamate and saccharin, are under suspicion as potential bladder carcinogens, although the equivocal nature of the experimental findings has elicited continual controversy and prompted the initiation of further animal work on the two compounds (*Cited in F.C.T.* 1976, **14**, 213).

One novel experimental approach to the problem

has been tried by the authors of the paper cited above, who used a sensitive rat model designed to detect weak bladder carcinogens or co-carcinogens. In this system, the test compound is given to animals that have received a single initiating but non-carcinogenic dose of the carcinogen *N*-methyl-*N*-nitrosourea (MNU). In the experiments reported here, groups of SPF Wistar rats free from the bladder parasite *Trichosomoides crassicauda* were fed sodium cyclamate or sodium saccharin at levels of 1–2 g/kg/day and 2–4 g/kg/day, respectively, or were given a single ip injection of 200 mg cyclophosphamide/kg. For comparison, the three compounds were also tested as solitary carcinogens in rats not pre-treated with MNU. Saccharin and cyclamate, when fed to untreated rats, produced a bladder-tumour incidence of 4/253 and 3/228, respectively, the first tumours appearing after 80 wk. Cyclophosphamide failed to produce tumours when administered alone or to the MNU/rat model. Over half of the animals receiving sweeteners in addition to MNU developed bladder tumours (46/79 rats fed saccharin and 31/54 rats fed cyclamate), the tumours appearing from wk 10 onwards. Bladder calculi were found in many of the MNU-treated rats fed one of the sweeteners, but the authors discount the hypothesis that tumour development might be an indirect effect resulting from stone formation.

It is concluded that saccharin and cyclamate are weak bladder carcinogens, which exert a syncarcinogenic effect with MNU. The negative results with cyclophosphamide, a compound of known cytotoxicity to the bladder epithelium, serve, in the authors' opinion, to refute the possibility of false-positive results arising from a mechanism of carcinogenesis by non-specific bladder irritation.

The authors state that the MNU/rat model can be used to detect carcinogenic potential in compounds organotropic for the bladder more rapidly and with fewer animals than can be achieved by conventional methods of carcinogen testing.

[The possibility of tumour production by non-specific bladder irritation in this model has not been eliminated completely. The validity of comparing a negative result after a single ip injection of cyclophosphamide with positive results induced by the daily administration of very high doses of sweeteners in the diet is questionable.]

Fd Cosmet. Toxicol. Vol. 14, p. 369. Pergamon Press 1976. Printed in Great Britain

ANNOUNCEMENT

FRAGRANCE INDUSTRY CODE OF PRACTICE

National associations of fragrance manufacturers of several European countries—Belgium, France, Germany, Italy, The Netherlands, Spain, Switzerland and the UK—as well as of the United States and Japan, have formed the International Fragrance Association (IFRA) to deal with legislative and health matters relating to fragrance materials. A Code of Practice, drawn up for the industry by IFRA's Technical Advisory Committee and adopted unanimously by the member associations, specifies the difference between a fragrance ingredient, a fragrance compound (a blend of ingredients under a specific formula) and a fragrance material and lays down basic standards of good manufacturing practice relating to personnel, hygienic working conditions, storage, manufacturing operations, labelling, packaging and quality control.

It is stressed that fragrance materials must be used only in conditions under which they present no risk to health. For materials with a long history of use, the absence of unfavourable reports is considered a strong indication of safety, but it is emphasized that materials for which in-use experience is lacking should be used only after adequate evaluation. Minimum requirements for such materials are tests for acute oral toxicity, skin-irritation potential and skin-sensitization potential and, where relevant, for eye irritation or phototoxicity and photosensitization.

The Technical Advisory Committee collects and processes data relevant to the safety of fragrance ingredients and co-operates with the Research Institute for Fragrance Materials (RIFM) in the USA, operating a mutual exchange of information with the RIFM Expert Panel. On the basis of the considerations of the RIFM Experts and all other relevant data, this IFRA Committee establishes, updates and maintains industry guidelines for the safe use of fragrance ingredients, recommending in some cases that certain ingredients should not be used and in others that use levels and applications should be restricted.

This voluntary self-control by the fragrance industry is based on the acceptance of detailed prescriptions and standards for the manufacture of fragrance materials, on the testing of newly developed fragrance materials in accordance with the minimum test requirements and on the continuing re-examination of ingredients with a long history of use as new knowledge becomes available.

Fd Cosmet. Toxicol. Vol. 14, p. 371. Pergamon Press 1976. Printed in Great Britain

FORTHCOMING PAPERS

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

Studies on the metabolism of sucrose acetate isobutyrate in the rat and in man. By J. C. Phillips, J. Kingsnorth, I. Rowland, S. D. Gangolli and A. G. Lloyd.

Effects of butylated hydroxytoluene on *Tetrahymena pyriformis*. By J. G. Surak, R. L. Bradley, Jr., A. L. Branen, W. E. Ribelin and E. Shrago.

Long-term toxicity studies of sorbic acid in mice. By R. J. Hendy, J. Hardy, I. F. Gaunt, I. S. Kiss and K. R. Butterworth.

Long-term toxicity of parasorbic acid in rats. By P. L. Mason, I. F. Gaunt, J. Hardy, I. S. Kiss, K. R. Butterworth and S. D. Gangolli.

Long-term toxicity of parasorbic acid in mice. By P. L. Mason, I. F. Gaunt, J. Hardy, I. S. Kiss and K. R. Butterworth.

Pharmacokinetic profile of Diamidfos in rats. By M. W. Sauerhoff, J. P. Heeschen, R. A. Nyquist and W. H. Braun.

Short-term peroral toxicity of ethylidene gyromitrin in rabbits and chickens. By A. Niskanen, H. Pyysalo, E. Rimaila-Pärnänen and P. Hartikka.

Reproductive and peri- and postnatal studies with hexachlorophene. By G. L. Kennedy, Jr., S. H. Smith, J. B. Plant, M. L. Keplinger and J. C. Calandra.

The effect of surfactants upon rat peritoneal mast cells *in vitro*. By C. Prottey and T. F. M. Ferguson.

The effect of surfactants upon mammalian cells *in vitro*. By T. F. M. Ferguson and C. Prottey.

Histochemical and biochemical studies of chemically induced acute kidney damage in the rat. By R. C. Cottrell, C. E. Agrelo, S. D. Gangolli and P. Grasso.

Metabolism of ochratoxins A and B in the pig during early pregnancy and the accumulation in body tissues of ochratoxin A only. By D. S. P. Patterson, B. A. Roberts and B. J. Small. (Short paper)

Monographs on fragrance raw materials. By D. L. J. Opdyke.

Recent studies of lysinoalanine in alkali-treated proteins. By C. J. O'Donovan. (Review paper)

Fd Cosmet. Toxicol. Vol. 14. p. 373. Pergamon Press 1976. Printed in Great Britain

CORRIGENDUM

Volume 14 (1976)

p. 82, line 38: *For* 1975, **7,** 1) *read* 1975, **8,** 171).

Contents continued]

REVIEW SECTION

REVIEWS OF RECENT PUBLICATIONS 339

BOOK REVIEWS 341

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 347

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 355

ANNOUNCEMENT 369

FORTHCOMING PAPERS 371

CORRIGENDUM 373

Aims and Scope

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

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

Annals of Occupational Hygiene

European Journal of Cancer

Archives of Oral Biology

Health Physics

Atmospheric Environment

Journal of Aerosol Science

Biochemical Pharmacology

Journal of Neurochemistry

Chronic Diseases

Toxicon

Life Sciences

Each journal has an individual Information and Index Leaflet giving full details. Write now for any of these leaflets which interests you.

Instructions to Authors

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Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced*. At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal [abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*, 4th Ed., Butterworth & Co. (Publishers) Ltd. London], volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. I. Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum.* 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b etc. following the citation of the year:

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Footnotes. These as distinct from literature references should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § || ¶ in that order.

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