Food and Cosmetics Toxicology

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Contents

Volume 15 Number 1

RESEARCH SECTION

Long-term toxicity study of Emulsifier YN in the mouse (*I. F. Gaunt, K. R. Butterworth, P. Grasso and A. V. Ginocchio*) 1

A 2-year feeding study of instant coffees in rats. I. Body weight, food consumption, haematological parameters and plasma chemistry (*H.-P. Würzner, E. Lindström, L. Vuataz and H. Luginbühl*) 7

Hydrolysis of phthalate esters by the gastro-intestinal contents of the rat (*I. R. Rowland, R. C. Cottrell and J. C. Phillips*) 17

Effect of aflatoxin B_1, ochratoxin A and rubratoxin B on infant rats (*A. W. Hayes, J. A. Cain and B. G. Moore*) 23

Citrinin mycotoxicosis in the mouse (*W. H. Jordan, W. W. Carlton and G. A. Sansing*) 29

Effects of vitamin A on toxicity of hexachlorophene in the rat (*J. F. Hanig, J. M. Morrison, Jr., A. G. Darr and S. Krop*) 35

Antiperspirant aerosols: Airway responses following acute and subchronic exposure (*L. Diamond, S. Guttman, C. Chandler and T. Foster*) 39

Preliminary neurological evaluation of generalized weakness in zinc pyrithione-treated rats (*D. R. Snyder, E. J. Gralla, G. L. Coleman and J. H. Wedig*) 43

Absorption, excretion and tissue distribution of 2-pyridinethiol-1-oxide (*S. A. Ziller*) 49

Phototoxicity testing of fragrance raw materials (*P. D. Forbes, F. Urbach and R. E. Davies*) 55

SHORT PAPER

Non-teratogenicity of D- and L-goitrin in the rat (*K. S. Khera*) 61

REVIEW SECTION

REVIEWS OF RECENT PUBLICATIONS 63

BOOK REVIEWS 65

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 69

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 77

ANNOUNCEMENTS 89

FORTHCOMING PAPERS 91

Volume 15 Number 2

RESEARCH SECTION

Enhancing effect of phenobarbitone and butylated hydroxytoluene on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat (*C. Peraino, R. J. M. Fry, E. Staffeldt and J. P. Christopher*) 93

On the aetiology of gastric cancer: Mutagenicity of food extracts after incubation with nitrite (*H. Marquardt, F. Rufino and J. H. Weisburger*) 97

Absorption and metabolism of chlorinated fatty acids and triglycerides in rats (*H. M. Cunningham and G. A. Lawrence*) 101

Absorption and distribution studies on chlorinated oleic acid and extracts of chlorinated lipid and protein fractions of flour in rats (*H. M. Cunningham and G. A. Lawrence*) 105

Synthesis and properties of Nε-(DL-2-amino-2-carboxyethyl)-L-lysine, lysinoalanine (*J. C. Woodard, D. D. Short, C. E. Strattan and J. H. Duncan*) 109

Renal toxicity of Nε-(DL-2-amino-2-carboxyethyl)-L-lysine (lysinoalanine) in rats (*J. C. Woodard and D. D. Short*) 117

Inhalation of talc baby powder by hamsters (*A. P. Wehner, G. M. Zwicker, W. C. Cannon, C. R. Watson and W. W. Carlton*) 121

Evaluation of the corneal irritancy of test shampoos and detergents in various animal species (*L. L. Gershbein and J. E. McDonald*) 131

Serum alkaline phosphatase and microsomal enzymes in the beagle dog (*R. T. Keefe, P. M. Newberne and T. Myers*) 135

SHORT PAPERS

Inhibitory effect of butylated hydroxytoluene (BHT) on intestinal carcinogenesis in rats by azoxymethane (*E. K. Weisburger, R. P. Evarts and M. L. Wenk*) 139

Nitrate, nitrite, dimethylnitrosamine and *N*-nitrosopyrrolidine in some Chinese food products (*Y. Y. Fong and W. C. Chan*) 143

Effect of various dietary components on absorption and tissue distribution of orally administered inorganic tin in rats (*P. Fritsch, G. de Saint Blanquat and R. Derache*) 147

REVIEW SECTION

BOOK REVIEWS 151

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 153

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 157

MEETING ANNOUNCEMENTS 163

FORTHCOMING PAPERS 165

Volume 15 Number 3

RESEARCH SECTION

Phenolic antioxidants and the inhibition of hepatotoxicity from *N*-dimethylnitrosamine formed *in situ* in the rat stomach (*B. D. Astill and L. T. Mulligan*) 167

Regeneration of rat liver in the presence of essential oils and their components (*L. L. Gershbein*) 173

Placental and mammary transfer of chlorinated fatty acids in rats (*H. M. Cunningham and G. A. Lawrence*) 183

Effect of benomyl and benomyl hydrolysis products on *Tetrahymena pyriformis* (*P. W. Rankin, J. G. Surak and N. P. Thompson*) 187

Determination of polychlorinated dibenzofurans in tissues of patients with 'Yusho' (*J. Nagayama, Y. Masuda and M. Kuratsune*) 195

The fate of ochratoxin A in rats (*F. C. Chang and F. S. Chu*) 199

Metabolism and toxicity of halogenated carbanilides: Absorption, distribution and excretion of radioactivity from 3,4,4'-trichloro[^{14}C]carbanilide (TCC) and 3-trifluoromethyl-4,4'-dichloro[^{14}C]carbanilide (TFC) in rats (*R. A. Hiles*) 205

Pulmonary deposition, translocation and clearance of inhaled neutron-activated talc in hamsters (*A. P. Wehner, C. L. Wilkerson, W. C. Cannon, R. L. Buschboom and T. M. Tanner*) 213

SHORT PAPER

Mutagenic activity of hymenovin, a sesquiterpene lactone from Western bitterweed (*J. T. MacGregor*) 225

PRELIMINARY COMMUNICATION

Transplacental chronic toxicity test of carbaryl with nitrite in rats (*W. Lijinsky and H. W. Taylor*) 229

REVIEW SECTION

SHORT REVIEW

Quantitative aspects of human exposure to nitrosamines (*M. C. Archer and J. S. Wishnok*) 233

REVIEWS OF RECENT PUBLICATIONS 237

BOOK REVIEWS 239

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 243

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 251

LETTER TO THE EDITOR

Phototoxicity of fragrance materials (*P. D. Forbes, R. E. Davies and F. Urbach*) 265

FORTHCOMING PAPERS 267

Volume 15 Number 4

RESEARCH SECTION

Feeding tests in rats on mixtures of nitrite with secondary and tertiary amines of environmental importance (*W. Lijinski and H. W. Taylor*) 269

Primary and secondary amines in the human environment (*G. B. Neurath, M. Dünger, F. G. Pein, D. Ambrosius and O. Schreiber*) 275

Toxic synergism of methylmercury with sodium nitrite and ethylurea on reproduction and survival of progeny in rats (*J. E. Nixon*) 283

A 2-year feeding study of instant coffees in rats. II. Incidence and types of neoplasms (*H.-P. Würzner, E. Lindström, L. Vuataz and H. Luginbühl*) 289

Evaluation of acute and short-term administration of 2,4,5-trichlorophenoxyacetate with respect to renal proximal tubular transport (*F. J. Koschier and W. O. Berndt*) 297

Mycotoxins in foodstuffs. X. Production of citrinin by *Penicillium chrysogenum* in bread (*J. Reiss*) 303

Safety testing of alkyl polyethoxylate nonionic surfactants. I. Acute effects (*G. M. Benke, N. M. Brown, M. J. Walsh and R. B. Drotman*) 309

Safety testing of alkyl polyethoxylate nonionic surfactants. II. Subchronic studies (*N. M. Brown and G. M. Benke*) 319

Primary sensitization potentials of some halogenated salicylanilides and their cross-sensitivity in guinea-pigs (*C. W. Chung and A. L. Giles, Jr.*) 325

SHORT PAPERS

Ability of adult and foetal rat tissues to metabolize chlorinated fatty acids (*H. M. Cunningham and G. A. Lawrence*) 331

Excretion and metabolism of 2,4,5,2',5'-pentachlorobiphenyl in the squirrel monkey (*S. Holm*) 335

Teratogenic evaluation of piperonyl butoxide in the rat (*G. L. Kennedy, Jr., S. H. Smith, F. K. Konoshita, M. L. Keplinger and J. C. Calandra*) 337

REVIEW SECTION

BOOK REVIEWS 341

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 347

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 357

MEETING ANNOUNCEMENTS 373

NOTICE 375

FORTHCOMING PAPERS 377

Volume 15 Number 5

RESEARCH SECTION

One-year toxicity study of Orange G in the ferret (*P. G. Brantom, I. F. Gaunt and J. Hardy*) 379

Short-term toxicity of furfuryl mercaptan in rats (*J. C. Phillips, I. F. Gaunt, J. Hardy, I. S. Kiss, S. D. Gangolli and K. R. Butterworth*) 383

Short-term toxicity study of di-(2-ethylhexyl) phthalate in rats (*T. J. B. Gray, K. R. Butterworth, I. F. Gaunt, P. Grasso and S. D. Gangolli*) 389

Morphological changes in monkeys consuming a diet containing low levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*J. R. Allen, D. A. Barsotti, J. P. Van Miller, L. J. Abrahamson and J. J. Lalich*) 401

Mycotoxic diseases produced in mice by species of the *Aspergillus ochraceus* group (*J. L. Zimmermann, W. W. Carlton, J. Tuite and D. I. Fennell*) 411

Inhibition of yeast alcohol dehydrogenase by dehydroretronecine (*P. S. Sun, M.-T. S. Hsia, F. S. Chu and J. R. Allen*) 419

N-Nitrosodiethanolamine in cosmetics, lotions and shampoos (*T. Y. Fan, U. Goff, L. Song, D. H. Fine, G. P. Arsenault and K. Biemann*) 423

Effect of L-tryptophan on diethylnitrosamine and 3′-methyl-4-*N*-dimethylaminoazobenzene hepatocarcinogenesis (*R. P. Evarts and C. A. Brown*) 431

Volatile nitrosamines from ion-exchange resins (*T. A. Gough, K. S. Webb and M. F. McPhail*) 437

SHORT PAPERS

The presence of dimethyl- and diethylnitrosamines in deionized water (*W. Fiddler, J. W. Pensabene, R. C. Doerr and C. J. Dooley*) 441

Short-term toxicity study of *Paxillus involutus* in the rat (*L. Nieminen, K. Bjondahl, H. Ojanen and E. Ohenoja*) 445

Teratogenicity of 2,5-diaminotoluene, a hair-dye constituent, in mice (*M. Inouye and U. Murakami*) 447

Absorption of ingested talc by hamsters (*A. P. Wehner, T. M. Tanner and R. L. Buschbom*) 453

REVIEW SECTION

Criteria employed by the Expert Panel of FEMA for the GRAS evaluation of flavouring substances (*B. L. Oser and R. L. Hall*) 457

REVIEWS OF RECENT PUBLICATIONS 467

BOOK REVIEWS 469

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 475

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 487

FORTHCOMING PAPERS 501

CORRIGENDA 503

Volume 15 Number 6

RESEARCH SECTION

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

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

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

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

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

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

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

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

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

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

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

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

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

SHORT PAPER

Assessment of the acute toxicity and potential irritancy of hair dye constituents (*G. K. Lloyd, M. P. Liggett, S. R. Kynoch and R. E. Davies*) 607

MONOGRAPHS

Monographs on fragrance raw materials (*D. L. J. Opdyke*) 611

REVIEW SECTION

REVIEWS OF RECENT PUBLICATIONS 639

BOOK REVIEWS 641

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 645

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 649

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.

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A 2-year feeding study of instant coffees in rats. I. Body weight, food consumption, haematological parameters and plasma chemistry (*H.-P. Würzner, E. Lindström, L. Vuataz and H. Luginbühl*) 7

Hydrolysis of phthalate esters by the gastro-intestinal contents of the rat (*I. R. Rowland, R. C. Cottrell and J. C. Phillips*) 17

Effect of aflatoxin B_1, ochratoxin A and rubratoxin B on infant rats (*A. W. Hayes, J. A. Cain and B. G. Moore*) 23

Citrinin mycotoxicosis in the mouse (*W. H. Jordan, W. W. Carlton and G. A. Sansing*) 29

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Absorption, excretion and tissue distribution of 2-pyridinethiol-1-oxide (*S. A. Ziller*) 49

Phototoxicity testing of fragrance raw materials (*P. D. Forbes, F. Urbach and R. E. Davies*) 55

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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

Resolving the cyclamate question (p. 69); Species variation in methylmercury uptake (p. 70); Fluorocarbons near and far (p. 72); Short-term carcinogenicity tests: You pays your money.... (p. 74).

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

COLOURING MATTERS: The trial, error and metabolic fate of amaranth (p. 77)—FLAVOURINGS, SOLVENTS AND SWEETENERS: More metabolites of *d*-limonene (p. 77); Acetaldehyde and the rat foetus (p. 77); Alcohol and the foetal brain (p. 78)—PRESERVATIVES: AF-2 suspicions confirmed (p. 78); Nitrosamines out of the frying pan (p. 79)—PROCESSING AIDS: Polydimethylsiloxanes in the environment (p. 79)—AGRICULTURAL CHEMICALS: Metabolism of ethylene thiourea (p. 80); Filling in the HCB picture (p. 80); Naphthol in the river (p. 80)— PROCESSING AND PACKAGING CONTAMINANTS: Re-examination of the dioctyl tins? (p. 81)—MISCELLANEOUS CONTAMINANTS: Mercury and cell growth (p. 81)—THE CHEMICAL ENVIRONMENT: Aluminium toxicity in renal failure (p. 82); The nickel-plated lung (p. 82); Nickel and the epidermis (p. 83); Myocardial effects of acrylic bone cements (p. 83); Solvents, salves and sensitization (p. 84); TLV for formaldehyde questioned (p. 84); TDI and the lungs (p. 84); Triaryl phosphate poisoning in fish (p. 85); Mutagenicity of chlorinated ethylenes (p. 85); Behavioural effects of trichloroethylene (p. 85); Spray adhesives exonerated again (p. 86); Synthetic fibres in the air (p. 86)—BIOCHEMICAL PHARMACOLOGY: Comparative metabolism of drugs (p. 87)—TOXICOLOGY: Absorption of macromolecules in the newborn (p. 87)—PATHOLOGY: Starch powder reprieved? (p. 87)—CANCER RESEARCH: More nitrosamine studies (p. 88).

*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

LONG-TERM TOXICITY STUDY OF EMULSIFIER YN IN THE MOUSE

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Abstract—Emulsifier YN was fed to groups of 50 male and 50 female mice at dietary levels of 0 (control), 2 or 6% for 80 wk. These treatments had no adverse effect on mortality, haematology, organ weights, the incidence and severity of histopathological changes or the incidence of tumours. The no-untoward-effect level for YN in this study was thus 6%, equivalent to an intake by the mouse of approximately 8·5 g/kg/day.

INTRODUCTION

Emulsifier YN (subsequently referred to as YN) consists of a mixture of the ammonium salts of phosphatidic acids and triglycerides derived from rapeseed oil. It is produced by phosphorylation of the products of glycerolysis of partially-hardened rapeseed oil and final neutralization of the reaction mixture with ammonia. Six phosphatidic acids have been identified in the mixture before neutralization, namely mono-, bis-, and trisphosphatidic acids, lysophosphatidic acid (or the equivalent 1,2-cyclo- form), bisphosphatidyl-lysophosphatidic acid (or the equivalent 1,2-cyclo-form) and bisphosphatidyl-monophosphatidic acid. The relative contribution of each of these components to the mixture is not known and the percentage composition probably varies with different batches.

YN is used as an emulsifier and viscosity-reducing agent in the manufacture of chocolate. Natural lecithins have been used for this purpose, but only that from soya beans was available in sufficient quantity for commercial use and this had too strong a flavour for use in some types of chocolate. Hence YN was developed as a substitute and is used in chocolate at levels of 0·35–0·5%.

It has been shown (A. C. Frazer, unpublished report 1954) that diets containing 1 or 5% YN could be fed to rats for periods in excess of 45 wk with no effect. Using ^{32}P-labelled YN, Feuer (1967) concluded from *in vivo* studies in rats and from *in vitro* experiments, that 80% of the phosphate associated originally with the YN was excreted in the faeces. He also concluded that YN was degraded in the gastro-intestinal tract to yield phosphate, which was distributed throughout the body as part of the general phosphate pool.

The metabolic studies were extended (Phillips, Gaunt & Gangolli, 1975) to include *in vivo* studies in the mouse, guinea-pig and ferret, while the *in vitro* investigations were extended to include the hydrolytic effects of the liver and intestinal mucosa. These authors concluded that it was likely that the majority of the YN was hydrolysed in the intestine or by the intestinal mucosa to provide phosphate that could contribute to normal metabolic pathways. Since the liver was capable of hydrolysing any YN that was absorbed intact, it was considered unlikely that components of the emulsifier were likely to be incorporated into the body tissues.

In a short-term study in rats (Gaunt, Grasso & Gangolli, 1967), no untoward effects were detected in rats maintained on diets containing up to 6% YN. Extending this to a long-term study in rats using dietary levels of 2 and 6%, Brantom, Gaunt, Hardy, Grasso & Gangolli (1973) found no evidence that YN exerted any carcinogenic effect. It was found that thyroid weight was increased as a result of an increased incidence of parathyroid hyperplasia which, in turn, was thought to be due to the effects of spontaneous renal changes combined with an elevated intake of phosphate. Also there was a slight increase in the incidence of myocardial fibrosis which was associated with the parathyroid hyperplasia. The no-untoward-effect level for YN derived from this study in the rat was 6% of the diet, providing an intake of 3 g/kg/day. The present paper is the result of a similar study using the mouse as an alternative species. These long-term studies were carried out to meet the requirements of the report of the Joint FAO/WHO Expert Committee on Food Additives (1970).

EXPERIMENTAL

Materials. YN was supplied by Cadbury Ltd., Bournville, Birmingham, and conformed to the manufacturer's specification for YN for use in foodstuffs. It was described as consisting of a mixture of ammonium compounds of phosphatidic acids derived from rapeseed oil, and a proportion of triglycerides

from the partially-hardened oil. The impurities did not exceed 0·2% water, 2·5% matter insoluble in 40–60 petroleum ether, 0·2% inorganic matter insoluble in 40–60 petroleum ether, 42% unreacted triglyceride, 3·0–3·4% phosphorus, 2·5 ppm arsenic, 2 ppm lead and 2 ppm copper, and the pH of the mixture was between 6 and 8.

Animals and diet. The animals used in this study were obtained from A. Tuck & Son, Rayleigh, Essex. They were kept in an animal room maintained at 21 ± 1°C with a relative humidity of 50–60%, and were caged in groups of five. Food (Diet 41B meal) and water were provided *ad lib.* Experimental diets were prepared and the animals were housed and maintaned at Consultox Laboratories Ltd., Brent, London, under the joint supervision of Consultox and BIBRA scientists. The remainder of the investigations were carried out at the BIBRA Laboratories.

Experimental design and conduct. Groups of 50 male and 50 female mice were fed diets containing either 0 (control), 2 or 6% YN for 80 wk. The general condition and behaviour of the animals were monitored daily and any animal found to be in ill health was isolated, to be returned to its cage if its condition improved or killed if its condition deteriorated. A gross autopsy was conducted on all animals unless this was precluded by cannibalism or advanced autolysis. The animals surviving at wk 80 were killed by exsanguination from the aorta under barbiturate (Nembutal) anaesthesia following an overnight period without food. At autopsy any macroscopic abnormalities were noted and the brain, heart, liver, spleen, kidneys, stomach, small intestine, caecum and testes were weighed. Samples of these organs and of salivary gland, thyroid, thymus, adrenal glands, lymph nodes, pancreas, pituitary, ovaries, lung, trachea, aorta, skeletal muscle, colon, rectum, spinal cord, bone and uterus, together with any other tissue that appeared to be abnormal, were preserved in 10% buffered formalin. All tissues from the control animals and from those given diet containing 6% YN, together with all macroscopically abnormal tissues from the group fed diet containing 2% YN were embedded in paraffin wax and sections were stained with haematoxylin and eosin for microscopic examination. Smears of bone-marrow were prepared and stained but were not examined in the absence of effects in the haematological examinations.

Blood for haematological examination was collected from a caudal vein of ten male and ten female mice from each group at 13, 26 and 52 wk and from the aorta of all surviving mice during autopsy at the end of the study. Samples were examined for haemoglobin content and packed cell volume, and counts were made of erythrocytes and total leucocytes. Slides were prepared for the examination of red cell morphology, enumeration of the various types of leucocytes and for counts of reticulocytes, but the examination of these was confined to the control animals and those given 6% YN.

RESULTS

No abnormalities were seen in the behaviour of the mice until wk 68, when convulsions were noticed in one mouse from each group, including the controls, immediately following transfer to a fresh cage during routine cleaning. The convulsions persisted for 15–20 sec and were followed by copious salivation and hyper-irritability for approximately 10 min, by which time the animals appeared to have recovered. Similar convulsions were seen intermittently in the same circumstances in one or two animals in each group up to wk 74. During the last 6 wk of the study no abnormal behaviour was seen.

Approximately 33% of the males and 11% of the females died or were killed *in extremis* during the study, but there were no statistically significant differences between the groups in the numbers of animals dead at any time. In both sexes there were slightly fewer deaths among animals given 6% YN than among the controls, the numbers dead in the groups given 0, 2 and 6%, respectively, being 12, 8 and 4 males and 2, 3 and 2 females, at wk 70 and 22, 16 and 12 males and 6, 8 and 3 females at wk 80. In the haematological examination the only statistically significant differences between treated and control mice were seen in the erythrocyte counts at wk 13 (Table 1). These consisted of a lower value in the males given 2% YN but a higher value in the females given 6%. Occasional erythrocytes containing Heinz bodies were seen but the incidence in any one animal was less than 1% of the cells, while the incidence of affected animals did not differ in the various groups, including controls.

Statistically significant differences between the treated and control groups were found in the weights of the liver and kidneys of females (Table 2). The former organ was affected at the highest dietary level and the latter at the lower level (2%), and the differences remained significant when the weights were related to body weight. There were no comparable differences or trends in the males.

There were various histopathological changes (Table 3), but the severity and incidence of these were similar in all groups including the controls. Table 4 shows the incidence of tumours, the commonest being adenoma of the lungs. Most of the tumours were found either solely in control mice or with a similar incidence in treated and control animals. Only lymphosarcoma occurred in treated mice without a comparable finding in the controls but the incidence of this tumour was limited to one animal in each of the male groups given YN.

DISCUSSION

The observation that some of the mice showed convulsions when disturbed during cage-cleaning is unusual. Mice are normally hyperactive during such manipulations and any congenital tendency toward convulsions might be precipitated under these conditions. There was no histological evidence of damage to the central nervous system and since the convulsions were apparent in animals from the control as well as the treated groups, they could not be related to YN treatment.

The finding of slight changes in the numbers of circulating erythrocytes at wk 13 was not thought to be related to YN treatment. The changes were of opposite direction in the two sexes, and were not

Table 1. *Results of haematological investigations in mice fed diets containing 0, 2 or 6% Emulsifier YN for 80 wk*

Sex and dietary level (%)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC ($10^6/mm^3$)	Retics (% of HBC)	Leucocytes Total ($10^3/mm^3$)	Differential (%) N	E	L	M
				Wk 13						
Male										
0	10	17·2	49	8·37	—	21·3	33	0	66	1
2	10	15·8	50	7·06*	—	25·8	—	—	—	—
6	10	16·3	50	7·40	—	24·5	30	0	69	1
Female										
0	10	17·0	50	7·97	—	17·2	13	0	85	2
2	10	16·8	51	8·22	—	14·9	—	—	—	—
6	10	16·6	50	9·03*	—	17·6	19	0	80	1
				Wk 80						
Male										
0	25	15·2	44	8·16	2·6	16·4	28	0	71	1
2	34	14·9	44	8·05	—	14·6	—	—	—	—
6	37	14·6	45	7·78	1·5	13·0	33	1	66	0
Female										
0	41	15·4	48	8·76	2·9	14·1	26	1	73	0
2	41	15·2	49	8·63	—	13·2	—	—	—	—
6	47	15·1	44	8·66	2·7	12·6	28	1	71	0

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 (Student's t test) from the control value. $^*P < 0·05$. There were no significant differences between test and control groups at wk 26 and 52.

Basophils did not constitute more than 0·5% in any group.

Table 2. *Relative organ weights of mice fed diets containing 0, 2 or 6% Emulsifier YN for 80 wk*

Sex and dietary level (%)	No. of mice examined	Relative organ weight (g/100 g body weight) Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Testes	Terminal body weight (g)
Male											
0	25	1·23	0·55	4·27	0·46	1·58	1·20	5·79	0·56	0·75	34·5
2	32	1·28	0·56	4·10	0·29	1·55	1·63	5·80	0·55	0·69	34·1
6	37	1·30	0·57	4·15	0·33	1·52	1·33	5·91	0·53	0·73	33·8
Female											
0	42	1·52	0·54	4·56	0·39	1·27	1·34	7·04	0·69	—	28·3
2	40	1·54	0·53	4·73	0·43	1·38*	1·35	6·81	0·69	—	28·5
6	47	1·44	0·52	4·95*	0·51	1·30	1·40	7·00	0·63	—	29·2

Figures are means for the numbers of mice shown and those marked with an asterisk differ significantly ($P < 0·05$ by Student's t test) from the control value.

Table 3. *Incidence of histological findings (excluding tumours) in mice fed diets containing 0, 2 or 6% Emulsifier YN for 80 wk*

	No. of animals affected among					
	Males fed dietary levels (%) of			Females fed dietary levels (%) of		
	0	2	6	0	2	6
Tissue and type of abnormality / No. of mice examined....	25	34	37	41	41	47
Kidneys						
Glomerulonephrosis	6	3	12	3	5	4
Leucocyte foci	16	19	17	11	19	23
Cortical cysts	2	1	0	2	2	0
Protein casts	0	4	6	5	5	13
Heart and blood vessels						
Fibrosis	4	4	0	0	0	0
Leucocyte foci	2	0	1	0	1	4
Liver						
Leucocyte foci	13	13	12	22	23	28
Focal necrosis	6	6	8	6	5	14
Nodules	2	2	2	0	0	0
Vacuolated cells	0	2	2	0	2	1
Stomach and intestine						
Leucocyte infiltration	0	2	0	0	0	2
Skeletal muscle						
Sarcocystis muris infection	6	2	3	2	3	2
Lymph nodes						
Cystic	1	0	0	0	0	0
Haemorrhage	1	1	0	1	2	1
Prostate gland						
Foci of calcification	1	0	0	—	—	—
Spinal cord						
Swollen fibres	2	1	0	0	0	0
Salivary glands						
Leucocyte infiltration	0	2	0	0	0	0
Spleen						
Lymphoid hyperplasia	0	1	0	0	1	0
Thyroid						
Large vesicle containing colloid	2	2	1	2	2	3
Adrenals						
Glomerular-cell degeneration	2	0	0	0	0	0
Fascicular-cell degeneration	3	0	0	1	1	0
Downward growth of spindle cells	6	3	6	20	15	16
Cells containing ceroid	1	1	3	2	2	0
Brown degeneration	4	4	3	6	2	6
Lungs						
Leucocyte infiltration	4	3	8	3	5	10
Pneumonitis	4	6	3	7	7	12
Testes						
Partial atrophy	1	2	3	—	—	—
Calcified tubules	0	0	1	—	—	—
Ovaries						
Cysts	—	—	—	0	1	3
Cells containing ceroid	—	—	—	16	15	25
Haemorrhage	—	—	—	3	2	4
Uterus						
Cystic	—	—	—	22	20	23
Endometrial hyperplasia	—	—	—	15	20	24
Leucocyte infiltration	—	—	—	1	0	1

The figures represent the incidence of the findings among the numbers of mice shown. No incidence in a treated group differed significantly ($P > 0.05$ by chi-square test) from the control incidence.

Table 4. *Incidence of tumours in mice fed dietary levels of 0, 2 or 6% Emulsifier YN for 80 wk*

Organ and type of tumour		No. of animals with tumours among					
		Males given dietary level (%) of			Females given dietary level (%) of		
		0	2	6	0	2	6
	No. of mice examined...	25	34	37	41	41	47
Liver							
Adenoma		1	1	0	0	0	0
Lung							
Adenoma		2	4	2	1	0	1
Ovary							
Thecal-cell tumour		—	—	—	2	0	0
Adrenal							
Medullary-cell adenoma		0	1	0	1	1	0
Harderian gland							
Adenoma		1	0	1	0	0	0
Subcutaneous tissue							
Fibrosarcoma		0	0	0	1	0	0
Reticulo-endothelial system							
Lymphosarcoma		0	1	1	0	0	0

The figures represent the incidence of the tumour among the numbers of mice shown. No incidence in a treated group differed significantly ($P > 0{\cdot}05$ by chi-square test) from the control incidence.

encountered after more prolonged treatment. This does not suggest an effect of treatment.

The slightly increased weight of the liver in the females given the highest level of YN was not accompanied by any similar change in males or by any alteration in the incidence of abnormal histological findings in this organ. When expressed relative to body weight, the liver-weight increase, although statistically significant, was equivalent only to approximately 9% of the control value. In the absence of any supporting histological evidence of hepatic change, it seems unlikely that this isolated change in weight represents an adverse effect of YN. Similar arguments apply to the isolated increase in kidney weight in females given 2% YN. In addition, there was no evidence in this case of any dose relationship, since the renal weight in the animals on the higher dietary level was comparable with that of the control group. The feeding of diets containing YN did not influence the incidence or severity of the degenerative changes normally seen in mice of this age. Similarly benign and malignant tumours were found in both treated and control groups and were of types that have been shown to occur spontaneously in mice (Cloudman, 1956; Tucker & Baker, 1967).

The results of this study show no evidence that Emulsifier YN has any carcinogenic potential when fed to mice at levels up to 6% of the diet. This is in keeping with the earlier work in rats (Brantom *et al.* 1973). Since the isolated changes encountered were not considered to be related to treatment, the no-untoward-effect level for YN is 6% in the diet of mice. This level is equivalent to a daily intake of approximately 8·5 g/kg/day.

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A 2-YEAR FEEDING STUDY OF INSTANT COFFEES IN RATS. I. BODY WEIGHT, FOOD CONSUMPTION, HAEMATOLOGICAL PARAMETERS AND PLASMA CHEMISTRY

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Abstract—Regular and decaffeinated instant coffees were incorporated at a 6% level in a commercial standard diet fed to male and female rats for 2 yr. The coffee samples, extracted at different rates, were obtained from industrial and decaffeinated coffees, which were either freeze- or spray-dried. The average daily intake of coffee in this assay, not considering the first 4 wk, was about 2·9 g/kg for males and 3·5 g/kg for females, corresponding to about 80 cups for a 70-kg man and 70 cups for a 50-kg woman. Body weights of coffee-treated groups, especially in males, were, in general, lower than body weights of controls and were inversely proportional to the caffeine content of the coffee samples. Food intake in treated rats was similar to or higher than that in controls. Significant differences between certain groups in blood chemistry and haematology were noted, but they were not considered to indicate toxic or ill effects, an interpretation substantiated by the histological findings. Plasma cholesterol levels in both sexes consistently showed a positive correlation with caffeine intake. Caffeine developed no lipolytic action during this chronic feeding trial. For each sex the number of deaths was similar in all groups during and at the end of the assay, but death rates for males, including the controls, were higher than for females. Overall, the data collected in this chronic feeding trial suggest that the various instant coffee samples were tolerated by the rats and did not induce any toxic effects in this species.

INTRODUCTION

Although coffee drinking has become a world-wide habit, the historical questions about the effects of coffee drinking have not lost their actuality. Especially during the past decade epidemiological and biological studies on coffee have been intensified.

Through several retrospective and prospective epidemiological studies (Boston Collaborative Drug Surveillance Program, 1972; Bross & Tidings, 1973; Cole, 1971; Dawber, Kannel, & Corcdon, 1974; Framingham Heart Study, 1966; Friedman, Siegelaub & Seltzer, 1974; Hennekens, Drolette, Jesse, Davies & Hutchison, 1976; Heyden, Bartel, Cassel, Hames, Tyroler & Meier, 1972; Heyden, Tyroler, Cassel, Hames, Becker & Heiss, 1976; Higginson, 1966; Klatsky, Friedman & Siegelaub, 1973; Paul, 1968; Paul, Lepper, Phelan, Dupertuis, MacMilan, McKean & Park, 1963; Tibblin, Wilhelmsen & Werkö, 1975; Walker & Gregoratos, 1967) attempts have been made to correlate pathological conditions to the habit of coffee drinking and to the quantity of coffee consumed. None of these studies, however, has brought forth any conclusive evidence of ill effects resulting from coffee drinking. Epidemiological approaches alone cannot give reliable answers because of the difficulty of dissociating true coffee effects from the multiple interconnected environmental factors to which man is exposed and which render the selection of suitable populations and matching controls very difficult. Questions that cannot be answered and hypotheses that cannot be tested in epidemiological approaches need to be studied in animal experiments and extrapolations to man need to be made with due caution.

In most acute and chronic experiments, caffeine has been investigated as the outstanding pharmacodynamic principle of coffee (Beliles, 1972; Boyd, 1959 & 1969; Boyd, Dolman, Knight & Sheppard, 1965; Eichler & Mügge, 1932; Peters & Boyd, 1967a,b & 1971). To date, only two publications have dealt with the experimental long-term ingestion of coffee. Daubert (1967) reported on the effects resulting from the 2-yr feeding of soluble coffee, decaffeinated coffee, brewed coffee and caffeine using dietary levels of instant coffee ranging from 0 to 5% and the equivalent caffeine levels from 0 to 0·177%. There was no effect on growth at low levels, but an impairment was recorded with the highest levels of soluble coffee and caffeine during the initial period of trial. Histological changes occurred in the kidneys and livers of rats receiving caffeine at the highest level. Additionally, weights of the liver and kidney were increased in animals receiving soluble coffee and caffeine at the highest dietary level. Strubelt, Siegers, Breining & Steffen (1973) administered regular coffee and caffeine at two

dose levels (4 and 8 g coffee/100 ml water) corresponding to 35 and 65 mg caffeine/kg/day to male rats for 6–7 months. They found no toxic effect and no lipolytic action of coffee and caffeine. Body weights of the coffee-drinking rats were lower than those of controls or caffeine groups. Blood chemical and histological examinations revealed no ill effects at these dose levels. No information was given on tumour occurrence or morphological changes of organs other than liver and kidney.

The technology of instant-coffee production, involving extraction and drying as well as caffeine removal for the decaffeinated brands, has developed steadily during the last 30 yr, and our main objective was to test these different technological variables in a 2-yr feeding study in rats. The coffee samples tested included various instant coffees produced in industrial installations using different extraction techniques and drying processes and in some cases decaffeination. Effects on growth, food consumption, haematology and blood chemistry are reported in this paper. Tumour types and incidence, lesions in the cardiovascular system, especially arteriosclerosis, and pathological changes in other organs and tissues will be presented in subsequent papers.

EXPERIMENTAL

Instant-coffee samples. The instant-coffee samples used were extracted from a commercial mixture of Robusta and Arabica coffee beans with a low, medium or high extraction yield (Table 1). Decaffeinated samples were obtained using methylene chloride and the residual level of the solvent in the decaffeinated instant coffees was 1 ppm, established by the method of Hadorn & Suter (1958). Potential effects of the decaffeination process were evaluated by re-introducing caffeine to the coffee samples at the highest levels used in this trial.

Animals and maintenance. Outbred specified-pathogen-free Sprague–Dawley rats of both sexes were purchased from Süddeutsche Versuchstierfarm (Tuttlingen, Germany). After arrival in cages fitted with filters they were allowed an adaptation period of 8 days during which they were fed a standard commercial diet (Nafag 194 from Nafag AG, Gossau). On arrival all animals were marked by ear notching. During both the adaptation and the assay period the animals were housed singly under controlled barrier-sustained conditions in Makrolon cages type III with steamed dust-free hardwood shavings (Société Parisienne des Sciures, Pantin, France) as bedding material. Each sex was housed in a separate room kept at a temperature of 23 ± 1°C and a humidity of $55 \pm 5\%$. A 12 hr day–night cycle was applied. Experimenters were obliged to pass through an airlock–shower system and to wear sterile clothes, masks and gloves when working in the animal units. The experiment started in February.

Experimental groups and treatments. After the adaptation period the rats, which weighed initially just over 100 g were randomly distributed into 14 groups each with 40 males and 40 females. All instant-coffee samples (Table 1) were incorporated at a 6% level in the commercial diet. The animals had free access to food and tap-water (autoclaved at 80 and 125°C, respectively, for 30 min) throughout the assay.

Observations and methods of examination. The rats were checked daily for general appearance, clinical signs and survival. Body weights were recorded weekly. Food intake was recorded weekly during the first 4 wk of the assay and then weekly over a 4-wk period following months 3, 6 and 12.

After experimental periods of 3 and 12 months, ten rats of each sex and group were chosen randomly for haematology, blood chemistry determinations, autopsy and histopathological examination. For technical reasons one group a day was submitted to blood sampling and autopsy. At 24 months all surviving animals were examined in the same manner. After 16-hr (overnight) starvation, blood was drawn under CO_2–O_2 anaesthesia from the orbital venous plexus between 08.00 and 10.00 hr. Whole blood was examined for haemoglobin content and packed cell volume

Table 1. *Experimental groupings and data on instant-coffee samples*

		Data on instant-coffee samples administered		
Treatment group	Type of instant coffee administered	Extraction rate (%)	Way of drying	Caffeine content (g/100 g sample)
A	None (control)	—	—	—
B	Regular	23·0	Spray	5·9
C	Regular	37·4	Spray	4·1
D	Regular	50·2	Spray	2·9
E	Regular	50·2	Freeze	2·9
G	Decaffeinated	20·9	Spray	0·4
H	Decaffeinated	34·6	Spray	0·3
J	Decaffeinated	47·4	Spray	0·2
K	Decaffeinated	47·4	Freeze	0·2
L	Decaffeinated + caffeine	37·4	Spray	5·7
M	Decaffeinated + caffeine	50·2	Spray	5·9
O	Decaffeinated + caffeine	50·2	Freeze	5·9
P	Regular	47·0	Spray	4·3
S	Regular	47·0	Spray	3·9

and counts were made of red and white blood cells. Additionally, the prothrombin time was determined at 12 months. Plasma was analysed for the content of urea nitrogen, glucose, total protein, phospholipids, triglycerides, total cholesterol, free fatty acids and activity of glutamic–pyruvic transaminase and alkaline phosphatase.

Urine samples were collected from five male and five female rats of all groups at 3 and 12 months and from the survivors at 24 months. They were analysed for pH, haemoglobin, glucose and albumin using paper sticks. The sediment was examined microscopically for white and red blood cells and hyaline and granulated casts. In our experience urine analysis in rats has a low diagnostic value and has not contributed to the elucidation of treatment effects. We have refrained, therefore, from including these data in this paper.

An autopsy was performed immediately after killing. Gross changes were noted and photographic records of animals and organs were made if considered important. Standardized tissue samples were removed for microscopical examination. Autopsy results and microscopic findings will be presented in a subsequent paper.

Statistical treatment of the results. We were interested, first of all, in every paired comparison with a comparisonwise Type I error rate. To account for a highly significant heterogeneity in the within treatment variances, the version of the t-test proposed by Welch (1947) was applied. This procedure is satisfactory in case of hetero-scedasticity, as pointed out by Scheffé (1970). The levels of significance indicated in the tabulated results for blood chemistry and haematology are extracted from the entire sets of paired comparisons. The procedure developed by Dunnett (1964) to test several treatments against a control was not used because, for every parameter, the variances of many treatments were substantially smaller than the variance of the control. The treatment means were also regressed on the technological variables which are: X_1, caffeine content (%); X_2, actual extraction yield (not given in Table 1); X_3, drying. X_3 takes on the value 0 for freeze-drying and 1 for spray-drying.

The least-squares technique with the numbers of observations per treatment as weights, when necessary, was used systematically (LSI in Jacquez, Mather & Crawford, 1968). The weighted least-squares method was also applied, with weights equal to the reciprocal of the estimated variances of the treatment means (WLS in Jacquez *et al.* 1968). It happened that the coefficients of simple correlations appearing in the multiple regression outputs were a very good summary of these outputs. In addition to this, the LSI and WLS values of those coefficients were close to each other. Consequently, the results can be summarized as in Table 7, which gives, for the sake of clarity, only the sign and the level of significance of LSI coefficients of simple correlation between some parameters and the technological variables.

It should be noted that a test of significance

$$t = \frac{r}{\sqrt{1 - r^2}} \sqrt{v},$$

where v is the number of degrees of freedom, performed on a LSI coefficient of simple correlation implying X_3 (a binary variable) is equivalent to a traditional weighted t-test between the means of the treatment groups for $X_3 = 0$ and $X_3 = 1$.

RESULTS

General observations

Death of animals, especially males, occurred in all groups, particularly during the later part of the assay. The cumulative death rate (Table 2), including animals killed *in extremis*, does not reveal any obvious differences between the various groups. A discernible trend is a slightly lower death rate among males treated with the decaffeinated coffees, when compared either to control or to other treated groups. The major cause of illness and death was chronic progressive nephropathy, another important cause being neoplasia. The causes of illness and death will be discussed in a subsequent paper.

Table 2. *Cumulative number of deaths (including animals killed* in extremis) *in rats fed instant-coffee samples at 6% in their diet*

Treatment	Cumulative number of deaths in																			
	Males at month										Females at month									
	6	8	10	12	14	16	18	20	22	24	6	8	10	12	14	16	18	20	22	24
A	0	0	0	0	1	2	6	11	18	18	0	0	1	1	2	3	6	7	10	10
B	1	1	1	2	4	6	8	13	18	19	0	0	1	1	1	2	3	4	6	6
C	0	0	0	1	7	11	13	17	18	18	0	1	1	2	4	7	7	9	13	13
D	0	0	1	2	2	7	10	13	16	17	0	0	0	0	1	2	3	5	8	9
E	0	0	0	3	4	7	13	16	19	20	0	0	2	2	2	3	4	9	9	9
G	1	2	2	2	3	4	4	6	11	14	0	1	2	2	2	3	5	6	8	8
H	0	0	0	2	3	3	3	4	10	14	1	1	1	2	3	3	4	8	11	12
J	0	0	0	0	0	1	6	9	12	15	0	0	1	1	1	1	3	4	10	12
K	0	0	0	0	1	4	7	10	12	14	0	0	0	1	3	3	3	9	9	10
L	0	0	1	3	3	10	12	16	16	16	0	0	0	1	1	2	4	8	8	8
M	1	1	1	4	8	9	16	17	17	17	0	0	2	4	5	7	8	9	14	14
O	1	1	1	4	6	6	8	13	18	18	0	0	1	1	2	3	4	7	10	12
P	0	0	1	3	5	8	11	13	14	16	0	0	5	5	5	5	6	9	13	14
S	1	1	1	2	6	9	12	13	16	16	0	0	2	2	4	4	4	5	7	8

Table 3. *Mean body weights and average intakes of food and coffee for male rats fed instant-coffee samples at 6% in their diet*

Treatment group	Mean body weight (g) at month						Average‡ food intake (g/rat/day) during the 4-wk period following month				Average‡ coffee intake (g/kg/day) during the 4-wk period following month			
	0†	1	3	6	12	18	0†	3	6	12	0†	3	6	12
A	105	285	409	476	528	529	19·6	19·5	21·4	21·0	—	—	—	—
B	105	234***	354***	423***	439***	419***	16·0***	20·2	21·3	20·2	5·9	3·3	2·8	2·9
C	105	260***	377***	444***	455***	444**	18·8**	20·7**	22·0	21·3	6·2	3·2	2·9	2·8
D	101	277*	382***	438***	465***	468***	21·1***	19·7	21·1	20·5	6·6	3·0	2·7	2·6
E	101	272**	387**	451*	476***	457**	22·2***	22·2***	24·0***	23·7**	7·1	3·3	3·2	3·0
G	109	288	393**	456*	497*	497	21·2***	19·6	20·4*	19·9	6·3	2·9	2·6	2·4
H	103	283	397*	467	500*	508	20·3***	20·1	20·4*	19·5**	6·2	2·9	2·6	2·3
J	109	266***	377***	437***	482***	486*	19·5	18·8*	19·4***	19·6*	6·1	2·9	2·6	2·4
K	107	279	379***	442***	484***	471*	22·8***	19·6	20·1*	20·1	7·0	3·0	2·7	2·5
L	101	240***	360***	423***	443***	429***	17.3***	20·5**	22·2	20·9	6·3	3·3	3·1	3·0
M	103	247***	358***	421***	432***	428*	17·4***	20·4*	21·5	20·2	6·0	3·3	2·9	2·9
O	103	236***	350***	421***	438***	426***	17·5***	21·7***	22·4	20·9	6·3	3·5	2·9	3·0
P	105	268***	375***	431***	462***	455*	22·1***	22·7***	22·4	24·1**	7·1	3·5	3·0	3·1
S	109	278	383***	451**	481**	439***	23·4***	23·1***	25·1***	24·0**	7·1	3·5	3·2	3·0

†First day of assay.
‡Based on the weekly recorded individual body weights and intake of food of all the animals alive in that period.
Values marked with asterisks differ significantly from those of the control group (A): *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Owing to the considerable losses of animals during the last months of assay, adequate statistical comparisons at 24 months were not feasible, and body weight, food consumption and blood parameters for this period are not presented here.

In the results for plasma chemistry and haematology some significant differences between control and test groups may have been influenced by day-to-day variation arising from the arrangements for blood sampling described in the experimental procedure.

Growth, food consumption and coffee intake

Mean body weights of males of all treated groups (Table 3) remained, in general, significantly lower than that of control males. In addition, a significant ($P < 0{\cdot}01$ to $P < 0{\cdot}001$) negative correlation was evident between body weight and caffeine content at every period examined. In females this effect was considerably weaker and no significant correlation between body weight and caffeine content was observed (Table 4).

Table 4. *Mean body weights and average intake of food and coffee for female rats fed instant-coffee samples at 6% in their diet*

Treatment group	Mean body weight (g) at month						Average‡ food intake (g/rat/day) during the 4-wk period following month				Average‡ coffee intake (g/kg/day) during the 4-wk period following month			
	0†	1	3	6	12	18	0†	3	6	12	0†	3	6	12
A	103	191	249	287	299	303	14·5	14·2	17·0	14·8	—	—	—	—
B	104	178***	232***	269**	282**	281***	13·8***	14·7	16·6	15·6	5·7	3·7	3·7	3·4
C	103	184	245	280	299	290*	14·9*	15·3***	17·6	17·4***	5·9	3·7	3·6	3·5
D	101	187	247	284	307	305	15·0**	15·3***	17·1	16·9***	5·8	3·6	3·6	3·3
E	101	183	244	278	291	295**	16·0***	15·9***	18·5*	17·4***	6·4	3·8	3·7	3·6
G	107	189	241*	273**	290	303	15·0**	14·6	16·9	15·5	5·9	3·5	3·6	3·2
H	106	192	246	278	286*	294	15·1**	14·7*	16·0	14·8	5·8	3·5	3·4	3·1
J	106	186	241*	273*	281**	291	15·4***	13·8	14·4***	14·9	6·0	3·4	3·2	3·2
K	105	191	236***	267***	270***	286	17·2***	14·3	15·0**	14·6	6·7	3·5	3·4	3·2
L	101	177**	232***	271**	279**	279***	14·5	14·6	15·9	16·1*	6·1	3·6	3·5	3·5
M	103	186*	240**	280	297	290	14·8	15·3***	16·9	16·2	6·0	3·7	3·6	3·4
O	102	179***	235***	273**	281**	283***	14·3	15·7***	16·3	16·7***	6·0	3·8	3·5	3·4
P	104	190	245	277	296	303	16·2***	16·5***	16·6	17·6***	6·4	3·9	3·5	3·6
S	105	198	251	287	303	311*	17·8***	16·6***	17·7	17·2***	6·9	3·9	3·4	3·5

†First day of assay.
‡Based on the weekly recorded individual body weights and intake of food of all the animals alive in that period.
Values marked with asterisks differ significantly from those of the control group (A): *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Food consumption during the initial period of the trial was lowest in male groups receiving coffee samples containing the highest amount of caffeine (Table 3). On the other hand, food consumption similar to or higher than that in the control group was recorded in groups receiving coffee samples of lower caffeine content. During this period a negative correlation ($P < 0.05$) existed between food consumption and caffeine content. Later the situation was reversed and in all groups treated with regular-coffee samples the food consumption was similar to or higher than that in the control group. In groups treated with decaffeinated samples, food consumption similar to or lower than that in the control group were observed. A positive correlation (not significant) with the caffeine content was noted during these periods. Food consumption in treated females, in general, was similar to or higher than that in controls. During the first 4 wk, however, as in the case of the males, it was negatively (not significantly) correlated with caffeine content. Subsequently this correlation became positive (not significant).

The average daily intake of coffee/kg body weight was higher in both sexes during the first 4 wk of the experiment (Tables 3 & 4), after which it stabilized rapidly to a lower, fairly constant level. This was understandable because during the early period of rapid growth the animals consumed more food, expressed as g/kg body weight, than later when growth had slowed down.

Extraction rate or method of drying had no significant influence on growth or food consumption.

Plasma chemistry

Although, there were some significant differences between treated and control groups in the results of plasma analyses (Tables 5 & 6), no relationships between treatment and adverse effects was apparent.

In contrast to the 3-month results, at 12 months unusually high blood urea nitrogen values were observed in two male rats (C78 and E64) and in one female (B37), and histopathological examination of the kidneys confirmed in these animals an advanced stage of chronic progressive nephropathy, a common disease in aged rats irrespective of their treatment. Under these circumstances we replaced each of these outliers by the mean of the remaining nine values of the respective group (see footnotes Tables 5 & 6). Additionally, in a single female rat (B59), besides an exceptionally high value for triglycerides, a high value for cholesterol was found. The triglyceride value alone was replaced by the mean of the remaining nine for statistical treatment (see footnote Table 6). We noted in this case, too, advanced chronic progressive nephropathy as well as a moderate level of cardiac scarring.

The significant correlations observed between plasma parameters and technological variables are given in the Table 7. Caffeine content is the technological variable that influenced several parameters. A consistent observation made in male and female rats was the elevation of plasma cholesterol level as the caffeine content increased. This correlation was significant in the two sexes at both 3 and 12 months. Plasma phospholipids showed a similar trend but this was significant only in females at 12 months. Also triglyceride levels tended to increase with increasing caffeine content at 3 months but this was significant only in females. In contrast to these lipid fractions, free fatty acid levels were lower in regular-coffee-treated animals than in controls. A significant negative correlation between this parameter and caffeine content existed in males at 12 months.

Haematology

Results are presented in Tables 8 and 9. The differences between control and treated groups, observed in both sexes, are biologically of no significance. In females of treated groups, except for the group S, the white blood cell counts were consistently lower than in controls. This, however, cannot be interpreted as being treatment-induced since in simultaneous generation studies, with animals of the same strain and age, control values as low as $3.0 \times 10^3/mm^3$ were recorded. It only substantiates the commonly accepted view that variations in the white blood cell counts have to be interpreted with caution.

Occasional significant correlations between haematological parameters and various technological variables were noticed (Table 7). An interesting observation is the negative correlation between prothrombin time at 12 months and extraction rate. Further studies are needed to explain this observation.

DISCUSSION

Instant coffees, regular or decaffeinated, ingested by the rats in large amounts caused no toxic effect. The average daily intake of instant coffee was approximately 2·9 g/kg in males and 3·5 g/kg in females if the initial period of observation is not taken into account. Supposing that a cup of instant coffee contains 2·5 g of coffee solids, the average amount of coffee consumed by the rats, on a body-weight basis, would correspond to about 80 cups of coffee/day for a man of 70 kg and 70 cups/day for a woman of 50 kg. According to the Pan American Coffee Bureau, the average daily consumption of instant coffee in the USA was 2·78 cups in 1969 and 2·77 cups in 1971, calculated per instant-coffee drinker.

Significantly lower growth rates, especially of males, in treated groups, combined with a food consumption similar to or higher than that of controls, are consistent with unpublished observations made in this laboratory and with the results of Strubelt *et al.* (1973) and Daubert (1967). As shown by the correlation analysis, caffeine played a role in the reduction of growth rates of animals fed regular coffee. Miller, Stock & Stewart (1974) suggested that caffeine induces thermogenic effects; a dose of 250 mg caffeine induced a marked increase in oxygen consumption in fasted and non-fasted human subjects. In an unpublished study made in this laboratory, rats were allowed to choose freely between a standard diet and the same diet containing 6% instant coffee as in this assay. The animals accepted both diets readily and consumed more food altogether than did control animals, although their body weights remained comparable to those of the controls. Therefore, the lower growth rates in this study cannot be interpreted as toxic effects but can, at least partly, be attributed to the thermogenic effect of caffeine.

Table 5. *Plasma chemical results for male rats fed instant-coffee samples at 6% in their diet*

Treatment group	Month...	BUN (mg/100 ml)		Glucose (mg/100 ml)		TPROT (g/100 ml)		PLIP (mg/100 ml)		TRIG (mg/100 ml)		TCHOL (mg/100 ml)		FFA (μeq/litre)		GPT (μmol/min/litre)		AP (μmol/min/litre)	
		3	12	3	12	3	12	3	12	3	12	3	12	3	12	3	12	3	12
A		19·5	17·9	139	121	6·8	6·6	129	284	115	230	87	229	583	504	10·2	11·2	27·7	21·9
B		18·1*	21·3	117	110*	6·3**	6·8	149*	339	127	217	114***	295	621	430	10·9	14·2	27·8	28·8**
C		16·4***	20·5‡	129	124	6·8	6·9	170**	357*	97*	253	109**	337*	629	383*	9·8	11·6	24·3	22·2
D		14·3***	14·8*	127	120	6·5	7·1*	151	301	102	220	93	290*	526	564	9·9	17·9	20·2**	21·8
E		14·1***	29·6*§	117	116	6·6	7·3*	80***	440***	110	325	109*	359***	621	510	10·0	15·3	23·3*	29·7*
G		19·6	17·2	122	111*	6·5**	7·9***	216*	397**	124	349*	97	332*	631	659**	10·2	12·3	18·8***	25·7
H		18·8	18·1	123	122	6·6	7·8***	151	356*	92**	294	90	283	666*†	679***	10·7	11·1	30·6	26·7**
J		16·8***	21·1	119	127	6·7	7·7***	116*	281	118	182	80	229	569	621*	8·8	13·9	27·2	36·6***
K		17·5**	16·0	120	123	6·7	7·4	140	295	106	267	103*	241	694**	574	7·7***	13·7	30·6	25·7
L		14·7***	22·8**	128	103***	6·8	7·8***	190**	481**	123	288	145***	437**	497*	509	9·4	12·7	26·8	16·7**
M		17·5*	22·0*	126	109**	6·7	7·8***	180*	433***	150*	250	153**	387***	588	420	11·9*	15·9*	26·4	18·3
O		18·6	25·2*	121	111*	6·7	7·8***	161*	416**	116	300	139***	366**	724*	512	11·2†	13·4	27·6	27·0*
P		17·2*	14·0**	134	119	6·6	7·4**	159	373*	135	232	134*	305	579	564	9·3	10·5	33·7**	16·4***
S		18·9	15·6	145	110*	6·6	7·5**	155	330	128	250	129*	297*	539	553	9·8	15·1*	23·6*	18·3*

BUN = Blood urea nitrogen PLIP = Phospholipids TCHOL = Total cholesterol GPT = Glutamic–pyruvic transaminase
TPROT = Total protein TRIG = Triglycerides FFA = Free fatty acids AP = Alkaline phosphatase

†Mean of nine animals (not enough sample).
‡One value of 145·5 mg/100 ml (animal C 78) has been eliminated from the mean as an outlier and replaced by the mean of the remaining nine values.
§One value of 174·0 mg/100 ml (animal E 64) has been eliminated from the mean as an outlier and replaced by the mean of the remaining nine values.
Values are means for ten animals and those marked with asterisks differ significantly from those of the control group (A): $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Table 6. *Plasma chemical results for female rats fed instant-coffee samples at 6% in their diet*

Treatment group	BUN (mg/100 ml)		Glucose (mg/100 ml)		TPROT (g/100 ml)		PLIP (mg/100 ml)		TRIG (mg/100 ml)		TCHOL (mg/100 ml)		FFA (μeq/litre)		GPT (μmol/min/litre)		AP (μmol/min/litre)	
Month...	3	12	3	12	3	12	3	12	3	12	3	12	3	12	3	12	3	12
A	18·7	17·8	138	116	6·8	7·7	148	222	106†	163	77†	128	693	866	7·6	9·1	18·1	20·7
B	15·5**	18·2†	101***	111*	6·3***	7·4	196***	341***	149***	190§	124***	278*	739	749**	6·6*	10·0	17·3	20·7
C	17·6	16·3	107***	107*	6·5*	7·5	203***	307*	129**	147	114***†	199**	710†	714***	6·6*	8·5	15·2*	16·3
D	16·0**	15·1**	115**	108**	6·7	7·2***	190***	248	117	146	103**	175**	555*	608***	7·5	9·4	10·7***	18·3
E	15·7**	17·8	110***	108	6·7	7·2**	104***	270*	120*	125	103***	169**	767	633***	7·4	8·8	15·6	21·6
G	20·2	17·9	123	112	6·7	8·1**	203	263*	125*	168	88*	147	755	993*	8·5	9·6	18·8	24·9
H	16·8	19·0	118*	109**	6·8	8·0*	169	247	93	180	82	136	792	868	7·9	9·7	18·8	27·2
J	15·6**	15·8*	111**	107***	6·9	8·1**	150	238	127**	123	88*	126	787	815	7·7	7·9*	15·0*	30·8**
K	15·4**	18·1	119*	117	6·7	7·9	147	219	109	148	86	135	647	865	7·2	9·5	17·5	20·8
L	15·1**	14·8**	107***	114	6·9	8·2***	215***	341**	118	158	125***	219***	808	873	8·4†	9·1	15·0*	12·9***
M	18·1	18·3	135	114	6·7	7·6	208***	277	137**	137	140***	180*	586	771*	10·0**	9·2	14·7*	12·0***
O	17·1	18·2	112**	116	6·7	8·1*	193***	300***	128**	113	133***	190***	686	787*	8·5	9·6	12·8**	15·8*
P	14·6***	15·0*	111**	109*	6·7	7·8	164	360*	135**	195	115***	215*	685	908	7·2	9·4	19·0	13·9**
S	15·1***	14·4***	122*	103***	6·7	7·9	157	290***	107	131	92	195***	549**	855	8·4	8·0	11·8***	14·9*

BUN = Blood urea nitrogen PLIP = Phospholipids TCHOL = Total cholesterol GPT = Glutamic-pyruvic transaminase
TPROT = Total protein TRIG = Triglycerides FFA = Free fatty acids AP = Alkaline phosphatase

†Mean of nine animals (not enough sample).
‡One value of 118·5 mg (animal B 37) has been eliminated from the mean as an outlier and replaced by the mean of the remaining nine values.
§One value of 1295 mg (animal B 59) has been eliminated from the mean as an outlier and replaced by the mean of the remaining nine values.
Values are means for ten animals and those marked with asterisks differ significantly from those of the control group (A): $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

Table 7. *Simple correlation of technological variables with plasma chemistry and haematology*

Technological variable	Blood taken at month	Plasma chemistry: GLUC	PLIP	TRIG	TCHOL		FFA	AP	Haematology: HB	HTCR	WBC		PTT	
		M	F	F	M	F	M	F	M	M	M	F	M	F
Caffeine content	3			(+)*	(+)***	(+)***				(+)***	(−)*			
	12	(−)*	(+)**		(+)*	(+)***	(−)**	(−)***	(−)*		(+)*			
Extraction yield	12			(−)*								(+)*	(−)**	(−)*
Way of drying	3						(+)*							

GLUC = Glucose PLIP = Phospholipids TRIG = Triglycerides TCHOL = Total cholesterol FFA = Free fatty acids
AP = Alkaline phosphatase HB = Haemoglobin HTCR = Haematocrit WBC = White blood cells
PTT = Prothrombin time (measured only at 12 months)

The sign of correlation is shown as (+) or (−) and the significance level of the correlation coefficient with respect to zero is indicated by asterisks: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

Table 8. *Haematological results for male rats fed instant-coffee samples at 6% in their diet*

Treatment group	Red blood cells ($10^6/mm^3$)		Haemoglobin (g/100 ml)		Haematrocrit (%)		White blood cells ($10^3/mm^3$)		Prothrombin time (sec)
Month...	3	12	3	12	3	12	3	12	12
A	6·61	6·73	17·4	16·1	53	47	11·8	12·3	31
B	7·02*†	6·05**†	17·6†	16·6†	53†	50†	8·4**†	8·6***†	52
C	6·79	6·99	17·1	16·1	54	49*	10·7	13·9	29
D	7·16***	6·94	17·8	16·4	53	50**	7·1***	8·9***	42
E	6·76†	6·22**	17·8†	17·0**	53†	51***	9·9*†	10·8*	21
G	6·94	6·66	17·5	17·1*	52	52***	15·6***	9·4***	43
H	6·77	6·73	16·9	16·8**	52	52***	13·3	10·2*	40
J	6·58	6·18**	17·1	16·5*	52	48	13·7*	7·3***	27
K	6·91	6·75	16·8*	16·7	52	50**	14·9***	8·7***	25
L	6·97*	6·90	17·3	16·4	53	50	11·9	11·1	22
M	6·43	6·46	17·3	16·3	54	50	11·7	12·0	21
O	6·98*†	6·27	17·7†	15·6	55†	50	10·5†	14·1*	24
P	6·68†	6·96	17·1†	16·4	53†	53***	10·8†	10·2**	29
S	6·29†	6·35	16·9†	16·7*	53†	54***	12·4†	12·0	24

†Mean of nine values (not enough sample).
Values are means of ten animals and those marked with asterisks differ significantly from those of the control group (A): $^*P < 0·05$; $^{**}P < 0·01$; $^{***}P < 0·001$.

The significantly higher mean plasma levels of blood urea nitrogen in some male groups at 12 months were related to chronic progressive nephropathy as confirmed by histopathological examination. The slightly lower plasma glucose levels in treated groups agree with the results by Strubelt *et al.* (1973), and a possible role of caffeine content is suggested by the negative correlation, observed in males at 12 months, between this technological variable and the plasma glucose level. That lower glucose values were noted also in groups given decaffeinated coffee indicates, however, an influence of another factor. Both plasma glutamic–pyruvic transaminase and alkaline phosphatase activities, regardless of isolated significant differences from controls, may be considered normal, a fact confirmed by histopathological examination of organs. Our transaminase results agree with those of Strubelt *et al.* (1973) who found no difference between control and treated groups for this parameter.

The consistent positive correlation between plasma cholesterol level and caffeine content of the instant coffees agrees with the results of Naismith, Akinyanju & Yudkin (1969), who fed coffee, decaffeinated coffee or caffeine to male rats for 54 days. The quantity of coffee/100 g diet was 2·3 g and that of caffeine 140 mg, a quantity equal to the caffeine content of the supplement of coffee. A significant elevation of plasma cholesterol level was observed in the coffee and caffeine groups but not in the decaffeinated-coffee

Table 9. *Haematological results for female rats fed instant-coffee samples at 6% in their diet*

Treatment group	Red blood cells ($10^6/mm^3$)		Haemoglobin (g/100 ml)		Haematrocrit (%)		White blood cells ($10^3/mm^3$)		Prothrombin time (sec)
Month...	3	12	3	12	3	12	3	12	12
A	6·51	6·26	15·9	14·9	49	49	10·1	7·0	35
B	5·80**	5·55†	15·6	14·1†	45**	45†	4·6***	5·4†	47
C	5·92**	5·90*	16·1	15·0	48	47	5·2***	9·0**	36
D	6·20	6·45	16·8**	15·2	49	47	5·1***	8·4*	24
E	6·04*	6·58	16·7**	15·6**	49	48	6·6***	11·6***	21*
G	5·71***	6·03	16·4*	15·7**	49	51	6·3***	7·1	29
H	5·75**	6·84**	16·6**	15·6**	49	48	7·2**	6·1	31
J	6·03*	5·89*	16·2	15·3	48	47	8·7*	7·0	28
K	4·97***	6·62‡	15·9	15·6*‡	48	47‡	7·1***	9·7***‡	33
L	4·98***	6·43	16·6*	16·5***	52**	50	9·1	9·2**	30
M	5·11***	6·29	16·4	16·2***	48	51*	7·5**	8·7**	31
O	6·24*	5·77	16·1	16·1***	49	51*	5·6***	9·3***	26
P	5·14***	5·63***	16·4*	15·2	48	49	5·6***	9·2**	25
S	5·33***	5·83**	15·9	15·5*	48	49	12·5**	11.0***	35

†Mean of nine animals (not enough sample).
‡Mean of seven animals (not enough sample).
Values are means for ten animals and those marked with asterisks differ significantly from those of the control group (A): $^*P < 0·05$; $^{**}P < 0·01$; $^{***}P < 0·001$.

group. On the other hand, Strubelt *et al.* (1973) found no effect on plasma cholesterol level after administering coffee in the drinking-water to male rats in two assays for 27 and 33 wk. The dose levels used by these authors were lower than those used by Naismith *et al.* (1969) and by ourselves. Based on these three assays it seems that plasma cholesterol level in rats may respond to different levels of caffeine. Differences in cholesterol metabolism between animal species, especially variations in absorption and excretion, as well as the influence of intestinal flora (Dietschy & Wilson, 1970; Portman, 1962; Sodhi, 1975) call for caution in any attempt to extrapolate cholesterol values from experimental animals to man. This is also substantiated by the results of Heyden *et al.* (1972) who found no differences in plasma cholesterol and triglyceride levels in male and female subjects consuming high and low amounts of coffee. Therefore, the present results have little direct relevance to man and, in addition, the amounts of coffee ingested were much higher than those consumed by man.

The changes in plasma free fatty acid, phospholipid and triglyceride levels were less consistent. It is interesting to note that no lipolytic activity of caffeine could be demonstrated, contrary to numerous observations from acute trials in animals and in man (references reviewed by Estler & Ammon, 1976). This observation is in agreement with that of Strubelt *et al.* (1973) from their chronic experiments. Thus, there seems to be a difference in response between acute and chronic assays. Neither the way of administration, in diet or in drinking-water, nor the quantity of caffeine ingested seem to undermine this conclusion. A reason for this difference may be that, in chronic assays, intake of test substance occurs in small repeated doses whereas in acute trials a high amount may be given as a single dose. Phospholipid values agree with those reported by Naismith *et al.* (1969), who found increased levels in groups that received coffee or caffeine but not in those receiving decaffeinated coffee. Caffeine possibly has an effect on this parameter as suggested by the correlation analysis in males at 12 months. The significantly elevated plasma levels of phospholipids in decaffeinated-coffee groups are, therefore, likely to be related to factors other than caffeine. Coffee, as administered in these long-term assays, seems to have had little, if any, effect on plasma triglyceride levels in rats.

In conclusion, chronic feeding of instant coffees to rats at a high dose level in their diets, did not cause any toxic effect. Extraction rate, method of drying and decaffeination all had little or no effect on the parameters studied. Caffeine content of the coffee samples was the only variable that influenced several parameters in this assay. This again is in confirmation of the view that caffeine is the outstanding physiologically active component in coffee.

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HYDROLYSIS OF PHTHALATE ESTERS BY THE GASTRO-INTESTINAL CONTENTS OF THE RAT

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Abstract—The rates of hydrolysis *in vitro* of six phthalate diesters, namely dimethyl, diethyl, di-*n*-butyl, dicyclohexyl, di-*n*-octyl and di-(2-ethylhexyl) phthalate, were greatest in the presence of rat small-intestine contents and much slower with caecal or stomach contents. Dimethyl, diethyl and di-*n*-butyl phthalate were metabolized much more quickly than the rest. Enzymes of animal rather than bacterial origin appeared to be involved. The product of metabolism in every case was the corresponding monoester, the identity of the metabolite being established by thin-layer chromatography and mass and infra-red spectroscopy. There was a correlation between the rate of metabolism and the acute toxicity of the diesters in the rat.

INTRODUCTION

Diesters of phthalic acid, which are extensively used as plasticizers, have been detected in rivers, lakes and coastal waters (Corcoran, 1973; Hites, 1973; Mayer, Stalling & Johnson, 1972), in soil (Cifrulak, 1969) and in the air (Thomas, 1973). Their almost ubiquitous presence in the environment has generated much concern about their possible hazard to health.

Acute toxicity of the phthalate esters studied is low (Krauskopf, 1973; Shibko & Blumenthal, 1973), but longer periods of administration of some of the esters to rats have led to liver enlargement, changes in hepatic enzyme activity and testicular changes (Carpenter, Weil & Smyth, 1965; Lake, Gangolli, Grasso & Lloyd, 1975; B.G. Lake and T.J.B. Gray, personal communication 1976).

Studies of the metabolism of phthalate esters in the rat have been confined mainly to di-(2-ethylhexyl) phthalate (DEHP). Albro, Thomas & Fishbein (1973) and Daniel & Bratt (1974) have characterized the urinary metabolites of orally administered DEHP and shown that they are all derivatives of mono-(2-ethylhexyl) phthalate and, in addition, Albro & Thomas (1973) have shown that lipases can metabolize DEHP to the monoester *in vitro*. Recently, Rowland (1974) demonstrated that DEHP is hydrolysed to mono-(2-ethylhexyl) phthalate by the gut contents of the rat *in vitro*. This partial hydrolysis appears to be a crucial step, not only in the metabolism of the diester, but also for its toxicity, since the hepatic changes produced by DEHP can be substantially reproduced by administering the monoester (Lake *et al.* 1975).

In view of the importance of the hydrolysis step, the previous study of DEHP metabolism by rat-gut contents (Rowland, 1974) has been extended to encompass a further five commonly used phthalate esters in order to compare their rates of metabolism and to determine whether any correlation exists between these rates and the toxicity of the phthalates.

EXPERIMENTAL

Materials. Dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), di-*n*-octyl phthalate (DOP) and dicyclohexyl phthalate (DCHP) were obtained from BP Chemicals International Ltd., Epsom, Surrey. [Carboxyl-^{14}C]-labelled phthalate diesters were prepared from [carbonyl-^{14}C]-phthalic anhydride by the method of Albro & Thomas (1973). Each diester migrated as a single radioactive zone on thin-layer plates of silica gel HF_{254} (E. Merck AG, Darmstadt) developed in *n*-hexane–ethyl acetate (90:10, v/v). Monoesters of phthalic acid were prepared and purified as described by Albro *et al.* (1973).

Bacitracin and neomycin sulphate were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, and streptomycin sulphate was purchased from Boehringer Corporation (London) Ltd.

Animals. Adult male Wistar rats, weighing approximately 250 g, were used in most experiments, although in some cases younger male and female rats (40–200 g) were used. All the animals were obtained from a specified-pathogen-free breeding colony.

Incubations with suspensions of gut contents and faeces. Suspensions (0·2 g/ml) of human faeces or the contents of the rat stomach, small intestine or caecum were prepared as described previously (Rowland, 1974), and 1-ml aliquots were incubated at 37°C for various periods with [^{14}C]-labelled diesters (0·03 μCi/ml; 1 mg/ml final concentration) under a stream of oxygen-free nitrogen. At the end of the incubation period the mixture was acidified to pH 2 with 5 N-hydrochloric acid, saturated with sodium chloride and extracted with 1 ml ethyl acetate.

In some experiments, the suspension of intestinal contents was incubated at 37°C for 90 min before addition of [^{14}C]DMP and, in others, the suspension was centrifuged (10,000 g for 5 min at 4°C) and the supernatant was passed through a membrane filter (0·45 μm pore size) before incubation with the diester. The effect of antibiotics on DMP metabolism was studied by adding bacitracin, neomycin and streptomycin (each at 1 mg/ml) either to the incubation mixture or to the intestinal suspension during the pre-incubation period (37°C for 90 min) described above.

Chromatography. The metabolites and any unchanged diester in the ethyl acetate extract were

separated by thin-layer chromatography using *n*-hexane–ethyl acetate (90:10, v/v) and the radioactivity in each of the zones was counted using a Nuclear Chicago Mark 1 liquid scintillation counter, as described previously (Rowland, 1974). The metabolites, which remained at the origin of these thin-layer plates were partially identified by their rates of migration in two other solvent systems: 95% ethanol–water–NH_4OH, 10:12:16, by vol. (Braun & Geenen, 1962) and chloroform–methanol–acetic acid, 143:7:2, by vol. (Albro *et al.* 1973).

Isolation and characterization of metabolites. Small-intestinal contents from the rat were incubated for 16 hr at 37°C with DMP, DEP, DBP, or DEHP (5 mg/ml) as described above. At the end of the incubation, the acidified mixture was extracted with an equal volume of toluene and the toluene extract was then extracted twice with an equal volume of 0·2 M-K_2CO_3 (leaving any unchanged diester in the toluene phase). The K_2CO_3 solution was acidified with 5 N-HCl and extracted with 1 vol. diethyl ether and the ether extract was concentrated using a rotary evaporator. The extract was applied to a column (20 × 1·5 cm) of silicic acid which was developed with chloroform–methanol (143:9, v/v). Fractions (5 ml) were collected, those absorbing at 260 nm were pooled and concentrated using a rotary evaporator, and the residue was redissolved in carbon tetrachloride.

The infra-red spectra of the metabolites and authentic monoethyl phthalates in carbon tetrachloride were recorded using a Pye-Unicam SP200 infra-red spectrometer, with NaCl cells of 0·5-mm path length.

Mass spectra were obtained on a V.G. Micromass 70/70F high resolution organic mass spectrometer in the electron ionization mode, using a direct insertion probe and an electron energy of 80 eV. Data were processed on a V.G. Data System 2040.

RESULTS

Rates of metabolism

The rate of metabolism of the phthalate diesters was fastest in the presence of the contents of the rat small intestine. In the presence of caecal contents, the rate of breakdown was considerably slower and, with the exception of DMP, metabolism of phthalates by stomach contents was negligible (Table 1). Human faeces were not very active in phthalate metabolism.

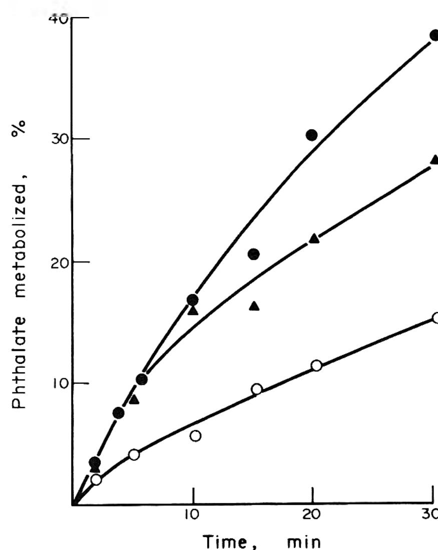

Fig. 1. Rate of metabolism of DMP (▲), DEP (○) and DBP (●) when incubated at a final concentration of 1 mg/ml with the contents of the rat small intestine (0·2 g/ml). Each point represents the mean value from three incubations. Further details are given in Table 1.

On the basis of their rates of breakdown by the content of the rat gut and by human faeces, the six phthalates fell into two groups: three of the esters—DMP, DEP and DBP—were metabolized rapidly, whereas DOP, DEHP and DCHP were metabolized much more slowly (Table 1). Of the latter group, DEHP was the fastest and DCHP by far the slowest to be metabolized.

The rates of metabolism of the three rapidly degraded diesters by the small-intestine contents over 30 min are shown in Fig. 1. The initial rates of metabolism of DBP and DMP were almost identical, although, over the 30-min period, rather more DBP (38%) than DMP (28%) was degraded. Over the same period of time, only about 12% of added DEP was metabolized and no degradation of DEHP, DOP or DCHP could be detected.

Table 1. *Metabolism of phthalate diesters by rat-gut contents and human faeces* in vitro

	Diester metabolized (%) in 16 hr by			
Phthalate	Stomach	Small intestine	Caecum	Human faeces
DMP	21·2 ± 1·1	61·1 ± 0·9	15·9 ± 0·4	8·3 ± 0·2
DEP	2·5 ± 0·2	36·4 ± 2·1	11·5 ± 0·5	3·0 ± 0·1
DBP	0·5 ± 0·07	80·8 ± 2·3	22·9 ± 1·3	6·2 ± 0·2
DCHP	0·4 ± 0·04	1·5 ± 0·1	0·3 ± 0·1	0·6 ± 0·1
DEHP	1·0 ± 0·2	22·1 ± 0·5	6·9 ± 1·0	0·6 ± 0·2
DOP	4·2 ± 2·2	11·1 ± 0·6	0·7 ± 0·1	NT

NT = Not tested

Gut contents (0·2 g/ml incubation mixture) were incubated at 37°C with [^{14}C] diester (1 mg/ml; 0·03 μCi/ml) and then the mixture was extracted with ethyl acetate. The percentage of diester metabolized to the monoester was calculated from the radioactivity remaining at the origin of chromatograms of the extract, developed in *n*-hexane–ethyl acetate. Each value represents the mean ± SEM of four incubations.

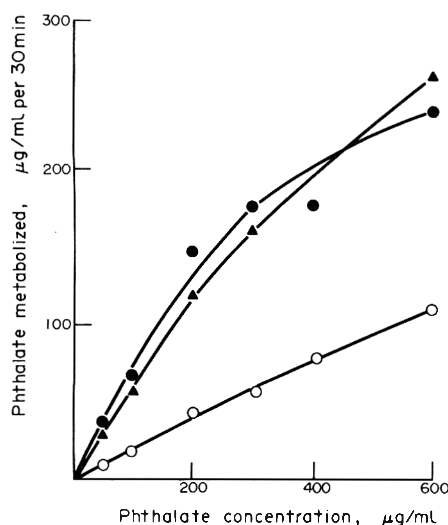

Fig. 2. Metabolism of DMP (▲), DEP (○) and DBP (●) when incubated at various concentrations with the contents of the rat small intestine (0·2 g/ml). Each point represents the mean value from three incubations. Further details are given in Table 1.

At low diester concentrations (less than 200 µg/ml) the amount of DMP, DEP and DBP degraded in 30 min was virtually directly proportional to the initial substrate concentration (Fig. 2); hence, the percentage degradation of these esters was more or less constant over this concentration range. For DMP and DBP, approximately 60 and 70%, respectively, was metabolized in 30 min at concentrations below 200 µg/ml.

Both the age and sex of the rats influenced the rate of metabolism of phthalates. Young male rats (26 days old) metabolized DBP much more slowly than animals only 7 days older. The latter metabolized DBP at a rate characteristic of the fully mature rats used in the other experiments (Table 2). A similar progression in the rate of metabolism with age was seen when DMP was used as the substrate. With female rats, however, age had little influence on the rate of degradation of DBP (Table 2). It is clear also that, among adults, females did not metabolize DBP as quickly as male rats.

The presence of antibiotics had no effect on the rate of metabolism of DMP by small-intestine contents (Table 3) even though the antibiotic mixture used (bacitracin, streptomycin and neomycin each in a concentration of 1 mg/ml) completely eliminates the intestinal bacterial flora in Wistar rats *in vivo* (I. R. Rowland, unpublished observations 1976). When the small-intestine contents were pre-incubated at 37°C for 90 min in the absence of DMP, their ability to degrade the diester was reduced by over 60% (Table 3), indicating that the enzymes involved in phthalate metabolism are labile *in vitro*. The presence of the antibiotic mixture during this pre-incubation period did not reduce the rate of DMP metabolism further (Table 3). Filtration of the suspension of small intestine contents through a membrane filter before incubation also reduced the rate of hydrolysis of DMP.

Identification of the metabolites

When the ethyl acetate extracts of incubation mixtures containing rat intestinal or caecal contents or human faeces and any one of the diesters were chromatographed on thin-layer plates using *n*-hexane–ethyl acetate, the radioactivity was distributed entirely between two zones. One part of the radioactivity remained at the origin and the other migrated at the same rate as the diester. The radioactive material at the origin was eluted and chromatographed further, using 95% ethanol–water–NH_4OH or chloroform–methanol–acetic acid. In each case, over 95% of the radioactivity migrated at the same rate as a sample of the monoester. No radioactivity was associated with the zone that migrated with an *o*-phthalic acid standard. Thus, rat gut contents or human faeces appear partially to hydrolyse the phthalate diesters to the corresponding monesters.

The product of metabolism of DMP, DEP, DBP, or DEHP by rat small-intestine contents was collected and purified so that it could be identified more rigorously using infra-red and mass spectroscopy.

The infra-red spectra of the metabolites of DMP, DEP, DBP and DEHP were identical to the spectra of the corresponding monoalkyl phthalates. The spectra of the monoesters were characterized by a strongly absorbing doublet at 1700 and 1720–1730 cm^{-1}, corresponding to a carbonyl group. The metabolites of the diesters each had this doublet in their spectra. The other major absorption peaks were at 2900 cm^{-1} and 1300 cm^{-1}.

For two of the phthalate esters (DBP and DEHP) mass spectra of the intestinal metabolites were recorded and compared to the spectra of authentic monoesters. The spectra of the metabolites and the monoalkyl phthalates each possessed a peak at *m/e* 149 corresponding to the protonated phthalic anhydride ion (Fig. 3). Other significant ions, derived from

Table 2. *Effect of age and sex on DBP metabolism by contents of rat small intestine*

Age (days)	DBP metabolized (%) in 30 min: Male	Female
26	7·8 ± 2·2	21·8 ± 0·7
33	34·5 ± 2·4	15·7 ± 2·5
40	34·4 ± 2·2	25·5 ± 3·3

Each value represents the mean ± SEM for three animals. For each animal, two estimations of the rate of metabolism were made and a mean calculated. Further details are given in Table 1.

Table 3. *Effect of various factors on the metabolism of [^{14}C]DMP by small-intestine contents*

Treatment of gut contents	DMP metabolized (%) in 30 min
None	23·3 ± 0·6
Antibiotics* present during incubation	24·7 ± 1·0
Filtered†	13·9 ± 0·6
Preincubated at 37°C for 90 min	8·1 ± 0·6
Preincubated at 37°C for 90 min with antibiotics*	6·9 ± 0·4

*Bacitracin, neomycin and streptomycin each at 1 mg/ml final concentration.
†Before incubation with DMP, the intestinal contents (in buffer–Ringers solution) were centrifuged (10,000 *g* for 5 min at 4°C) and the supernatant was passed through a membrane filter (0·45 μm pore size).
Each value represents the mean ±SEM of four incubations. Further details are given in Table 1.

that at *m/e* 149 and common to all the spectra, were at *m/e* 104 and 76.

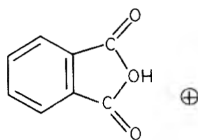

Fig. 3.

Peaks at *m/e* 57 and 43, corresponding to the ions $C_4H_9^+$ and $C_3H_7^+$, respectively, were present in the spectra of monobutyl phthalate and the metabolite of DBP.

Mono-(2-ethylhexyl) phthalate and the metabolite of DEHP both possessed peaks at *m/e* 278 corresponding to the parent ion (Fig. 4) and at *m/e* 113 ($C_8H_{17}^+$), 98, 70 and 59. Thus, the mass spectra of the metabolites of DEHP and DBP are consistent with their being mono-(2-ethylhexyl) phthalate and mono-*n*-butyl phthalate, respectively.

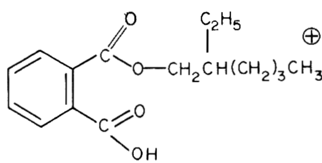

Fig. 4.

DISCUSSION

In a previous study of the metabolism of DEHP by rat-gut contents (Rowland, 1974), it was found that the rate of hydrolysis was faster in the presence of small-intestine contents than with caecal contents and that the rate in the presence of stomach contents was negligible. These findings have been confirmed in the present investigation using five more phthalate diesters, all of which were hydrolysed most rapidly by the contents of the small intestine.

The rates of hydrolysis of the six esters by intestinal contents varied considerably, from about 40% metabolized in 30 min for DBP, to 1% in 16 hr for DCHP at 1 mg/ml. The rate of metabolism was not related to the length of the side-chains of the diesters since although DMP was hydrolysed faster than DEP, the four-carbon side-chain of DBP was split off as quickly as the one-carbon chain in DMP. A more likely explanation of the differing rates of metabolism may be found in the water-solubilities of the diesters. Of the six diesters investigated, DMP and DBP are the most soluble (0·5 and 0·45 g/100 g water at 20°C, respectively) and DEP is less soluble (0·1 g/100 g water), whereas, of the three esters that were metabolized slowly, DCHP and DOP are insoluble and DEHP is slightly soluble (Fishbein & Albro, 1973).

At low concentrations, the percentage of phthalate metabolized was much higher than at 1 mg/ml, and a large proportion of added DMP or DBP was converted to the monoester in 30 min. Hence, when small amounts of such phthalates are ingested, it is likely they will be metabolized very rapidly in the gut.

The esterases involved in the partial hydrolysis of short-chain phthalate esters, such as DMP, appear to be mammalian in origin, since a mixture of antibiotics (known to inhibit all members of the gut flora) had no effect on the metabolism of DMP. With DEHP, however, it has been shown that the metabolism by intestinal contents can be reduced, although not completely eliminated, by adding antibiotics to the incubation mixture (Rowland, 1974). It is possible, therefore, that the hydrolysis of DEHP (and DOP and DCHP) is catalysed by esterases of bacterial and mammalian origin, whereas the rapid metabolism of DMP, DBP and DEP is catalysed predominantly by mammalian enzymes, most probably by extracellular lipases derived from the pancreas (Albro & Thomas, 1973; Daniel & Bratt, 1974) and enzymes associated with mucosal cells. The involvement of the latter is suggested by the reduction (almost 50%) in the rate of metabolism of DMP after filtration of the intestinal suspension.

The major contribution of mammalian enzymes to phthalate metabolism and their evident lability probably explains the low rates of phthalate hydrolysis by the caecal contents and by human faeces, in which the numbers of active intestinal mucosal cells must be small.

Although enzymes from various sources appear to be involved in the hydrolysis of phthalate diesters, the product of metabolism is the same in all cases, namely the corresponding monoester. The products

were identified by thin-layer chromatography using three different solvent systems and conclusive evidence was provided by infra-red spectroscopy and mass spectroscopy. It is interesting that only one metabolite is produced and that complete hydrolysis to phthalic acid does not occur. This is in accord with previous studies *in vivo* in the rat, which have shown that the urinary metabolites of DEHP are all derivatives of mono-(2-ethylhexyl) phthalate and that only very small quantities of phthalic acid are excreted (Albro *et al.* 1973; Daniel & Bratt, 1974).

It appears, therefore, that the intestinal hydrolysis of phthalate diesters is an important step in their metabolism and that ingested phthalates almost certainly undergo this reaction. Furthermore, at low phthalate concentrations, the percentage metabolized was much higher than at 1 mg/ml and a large proportion of added DMP and DBP was converted to the monoester in 30 min. Hence, when small amounts of diesters are ingested, it is likely that they will be hydrolysed very rapidly in the lumen of the gut.

The toxicological significance of the partial hydrolysis of phthalate esters in the gut contents may be assessed by comparing the rates of metabolism of the various diesters with their acute and chronic toxicities. When given orally to rats, those esters that were rapidly hydrolysed, namely DMP, DBP and DEP, were much more acutely toxic (on the basis of their LD_{50}) than DEHP, DOP and DCHP and, furthermore, of the former group, DEP was less toxic than DMP and DBP (Krauskopf, 1973; Shibko & Blumenthal, 1973). Thus, the rates of metabolism by intestinal contents and the acute toxicities of phthalate esters are closely correlated. No such correlation is apparent between rates of metabolism and chronic toxicity, although studies of the latter are few. When given orally to rats over a period of 1–2 yr, DEHP, DOP and DCHP were apparently more toxic than DMP, DEP and DBP (Shibko & Blumenthal, 1973). However, interpretation of the chronic toxicity data may be complicated by the fact that, under conditions of chronic administration, the rate of absorption of DEHP increased (Daniel & Bratt, 1974), probably because of an increase in the rate of conversion of DEHP to the monoester by the intestinal contents and mucosa (Rowland, 1974). A further toxic effect of certain phthalates, notably DEHP and DBP, is their ability to cause testicular atrophy in rats (Gray, Butterworth, Gaunt, Grasso & Gangolli, 1976; T.J.B. Gray, personal communication 1976). Although this does not appear to be related to the rate of hydrolysis of the diesters, it may be significant that both DBP and DEHP produce testicular atrophy only in those animals (rat and guinea-pig) whose intestinal contents can hydrolyse the diesters. In species such as the hamster, where little or no intestinal hydrolysis of DEP and DEHP occurs, testicular degeneration is not induced (T. J. B. Gray and I. R. Rowland, unpublished observation 1976).

It may be concluded, therefore, that the hydrolysis of phthalate diesters to the corresponding monoesters in the gut not only provides the initial step in the degradation of the diesters, but also may determine their degree of toxicity.

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EFFECT OF AFLATOXIN B_1, OCHRATOXIN A AND RUBRATOXIN B ON INFANT RATS

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Abstract—Oral LD_{50} values were determined in 24-hr-old Sprague–Dawley rats for aflatoxin B_1 (1·36 mg/kg), ochratoxin A (3·90 mg/kg) and rubratoxin B (6·38 mg/kg). Compared with the oral LD_{50} values in adults, the neonates showed an increased susceptibility to each mycotoxin. The calculated adult-to-neonate LD_{50} ratios were 9·2 for aflatoxin B_1, 6·5 for ochratoxin A and 49·2 for rubratoxin B. In simultaneous dosing experiments, the neonatal LD_{50} value of ochratoxin A in the presence of 5 mg rubratoxin B/kg was approximately 16 times below that for ochratoxin A alone. When the rubratoxin dose was five times smaller (1 mg/kg), the ochratoxin LD_{50} value was some ten times higher than that in the presence of the high rubratoxin dose. The neonatal LD_{50} value for rubratoxin B was decreased approximately fourfold in the presence of 2 mg ochratoxin/kg. Following administration of 5 mg rubratoxin/kg on day 1 and 4 mg aflatoxin B_1/kg on day 15, decreased weight gains were mediated by both agents rather than by either agent alone. A synergistic response was not demonstrated when aflatoxin B_1 was given on day 1 followed by rubratoxin B on day 15 or in experiments using ochratoxin followed by rubratoxin.

INTRODUCTION

While various secondary fungal metabolites are toxic to animals (Wilson & Hayes, 1973), moulds generally were not recognized as being responsible for outbreaks of disease until studies on the yellowed rice toxicoses in Japan (Kinosita, 1968) and on the occurrence of mould-induced alimentary toxic aleukia in Russia (Joffe, 1971) were reported in the 1950s. Concern about mouldy agricultural commodities was stimulated only after these incidents and after various other intoxications had been traced to food materials and feedstuffs contaminated with moulds. Worldwide attention was focussed on mycotoxicoses following the outbreak of "turkey X" disease in England in 1960 (Blount, 1961), while in the USA a high incidence of liver cancer in hatchery rainbow trout led to the discovery that aflatoxin-contaminated cottonseed meal had been used to feed the fish (Halver, 1965).

Observations that mycotoxins were carcinogenic, mutagenic and teratogenic in animal species added considerable impetus to research efforts on mycotoxins (Newberne, 1974). Incidence patterns of primary liver cancer in human populations in South-east Asia have been reported to correlate closely with contamination of dietary staples by aflatoxin (Shank, Bhamarapravati, Gordon & Wogan, 1972; Shank, Wogan, Gibson & Nondasuta, 1972). Recently, Wogan (1975) and his colleagues have correlated quantitative measurements of ingested aflatoxin in subpopulations in Thailand, Kenya and Mozambique with an elevated incidence of primary liver cancer, and the findings suggest that exposure to this carcinogen substantially elevates risk of this disease. Such studies, coupled with reports from the USA, Thailand, New Zealand and Czechoslovakia associating aflatoxin, fatty livers and the Reye-Johnson syndrome (Chaves-Carballo, Ellefson & Gomez, 1976) emphasize the need for investigation of the aflatoxins and other mycotoxins as food contaminants.

Although quantitative estimates of human exposure for other mycotoxins have not been sought systematically, a number of fungal metabolites are carcinogenic, mutagenic and teratogenic. Among such metabolites is ochratoxin A, first isolated from *Aspergillus ochraceus* growing on corn (van der Merwe, Steyn, Fourie, Scott & Theron, 1965). Ochratoxin is the agent responsible for the swine nephropathy reported from Denmark (Krogh, Hald, Englund, Rutqvist & Swahn, 1974) and is teratogenic to mice (Hayes, Hood & Lee, 1974) and hamsters (Hood, Naughton & Hayes, 1976). *Penicillium rubrum* was one of several toxigenic fungi isolated from cereal and legume products (Scott, 1965), and rubratoxin B, isolated from this mould, is mutagenic (Evans, Wilson & Harbison, 1975) as well as teratogenic (Hood, Innes & Hayes, 1973).

Because mycotoxins are foodborne, all age groups can be exposed. However, investigations of the mycotoxins, with the exception of aflatoxin (Wogan, 1966), have been concerned mainly with mature animals. Because of a lack of information on the response of young animals to mycotoxins, the toxicity of aflatoxin B_1, ochratoxin A and rubratoxin B to neonatal rats was studied. These studies were designed to demonstrate whether the neonate is more susceptible than the adult to the toxin and whether neonatal exposure leads to abnormal manifestations in the weanling or makes the animal more susceptible to a second mycotoxin given at the same time or several days later.

EXPERIMENTAL

Test materials. Rubratoxin was prepared by the method of Hayes & Wilson (1968), aflatoxin B_1 was purchased from Calbiochem, La Jolla, Cal., and ochratoxin A was supplied by Dr. Alex Ciegler, USDA, Peoria, Ill. Compound purity was established by thin-layer chromatography, melting point and infra-red and mass spectra. Mycotoxin stock solutions were prepared by dissolving individual toxins in 0·1 ml or less of a chloroform–methanol solution (2:1, v/v) and adding corn oil to give a final volume of 10 ml. The organic solvent was then removed *in vacuo* at room temperature and the solution was divided into five 2-ml aliquots, which were frozen at −85°C until used. This treatment did not affect the physical and chemical characteristics of the compounds or their toxicity to animals.

Animals and treatment. Adult male and female Sprague–Dawley rats (Charles River, Wilmington, Mass.) were maintained on standard laboratory chow and tap-water in rooms artificially lit for 12 hr/day. Each female was mated with two males overnight and then the females were caged separately. The day after delivery, pups were given, by intubation, one or two mycotoxins depending upon the experiment and then were returned immediately to the mother. Pups were handled only with gloves. Offspring were observed daily for signs of intoxication, morbidity and mortality, and weight gains were recorded.

The oral LD_{50} of aflatoxin B_1, ochratoxin A and rubratoxin B for neonate rats was determined by treating 24-hr-old pups in groups of at least ten with one of four dose levels of one of the mycotoxins. Control animals received only corn oil. Deaths were recorded daily for 7 days following toxin administration and the LD_{50} values and confidence intervals were calculated according to the procedure of Weil (1952).

A second study involved the simultaneous administration of two mycotoxins, each in the maximum dose producing no overt toxic effects when the toxin was given alone. In these experiments, 24-hr littermates were allocated in equal numbers to treated and control groups. The treated animals received mycotoxins by stomach tube, while control animals received corn oil that had been treated in the same way as the corn oil containing the toxin(s). The 7-day LD_{50} of one mycotoxin was determined in the presence of a constant dose of a second toxin.

Another study involving oral administration of one mycotoxin to 24-hr-old littermates and a second mycotoxin on day 15 was designed to test for delayed interactive effects. The pairs of mycotoxins tested in this way were rubratoxin/aflatoxin and ochratoxin/rubratoxin. The experiment was also carried out in reverse for the first of these toxin combinations. The number of deaths, average daily weight gains and liver-to-body weight ratios were followed for 30 days in both experiments.

Statistics. The various parameters were calculated as means ± SEM. Statistical significance of differences between mean values was determined by Student's t test (Steel & Torrie, 1960). Differences were considered significant at $P < 0.05$.

RESULTS

The neonatal 7-day oral LD_{50} values for aflatoxin B_1, ochratoxin A and rubratoxin B, together with the corresponding adult values, are presented in Table 1. The neonatal values were 1·36, 3·90 and 6·38 mg/kg for aflatoxin B_1, ochratoxin A and rubratoxin B, respectively. This LD_{50} value for aflatoxin B_1 compared favorably with that reported by Wogan (1966). The adult-to-neonate LD_{50} ratios based on our data for neonates and literature values for adults were 9·2, 6·5 and 49·2 for aflatoxin B_1, ochratoxin A and rubratoxin B, respectively. The response of neonates to these mycotoxins in comparison with the adult values demonstrated an increased susceptibility of young animals to each mycotoxin tested.

A dose response reflected in a decrease in weight gain in neonatal rats given ochratoxin A in a dose of 1, 2·5 or 5 mg/kg 24 hr after birth compared with that in control animals given corn oil is shown by the data presented in Fig. 1. All animals given 5·0 mg ochratoxin A/kg died within 14 days of treatment. Although the data are not presented, a similar dose response was observed in the weight gain of 24-hr-old pups treated with 0·5, 1·0 or 3·0 mg aflatoxin B_1/kg or 1, 3, 5 or 10 mg rubratoxin B/kg. Again, all pups given the highest doses (3 and 10 mg/kg for aflatoxin B_1 and rubratoxin B, respectively) died by day 14.

Table 1. *Adult and neonatal oral LD_{50} values for aflatoxin B_1, ochratoxin A and rubratoxin B in the rat*

Mycotoxin	Sex	Age	LD_{50} (mg/kg)	Solvent vehicle	Reference
Aflatoxin B_1	M	A	7·2	PG	Wogan, 1966
	F	A	17·9	PG	Wogan, 1966
	—	N	1·0	PG	Wogan, 1966
	—	N	1·36 (1·01–1·94)*	CO	Present report
Ochratoxin A	M	A	22·0	$NaHCO_3$	Purchase & Theron, 1968
	F	A	20·0	$NaHCO_3$	Purchase & Theron, 1968
	—	N	3·90 (3·21–4·61)*	CO	Present report
Rubratoxin B	M	A	400	DMSO	Wogan, Edwards & Newberne, 1971
	F	A	450	DMSO	Wogan *et al.* 1971
	—	N	6·38 (5·94–7·21)*	CO	Present report

A = Adult N = Neonate PG = Propylene glycol CO = Corn oil DMSO = Dimethylsulphoxide

*Animals received a single oral dose of mycotoxin. A minimum of ten animals was used at each dose level. The neonatal LD_{50} values, calculated according to the method of Weil (1952), include the fiducial limits.

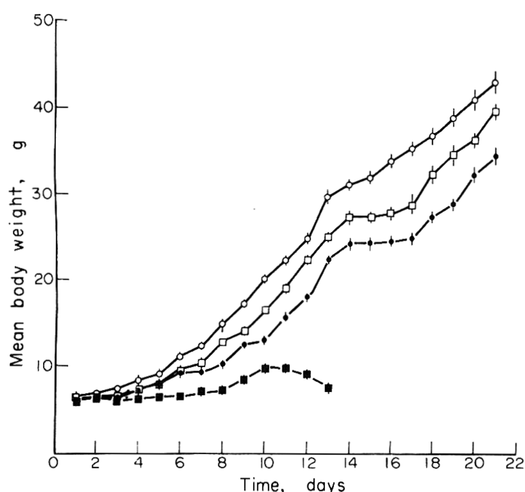

Fig. 1. Mean body weights of 24-hr-old rats given ochratoxin A in a single oral dose of 0 (control; ○), 1·0 (□), 2·5 (●) or 5·0 (■) mg/kg. Values are the means ± SEM for a minium of ten animals.

The LD_{50} for ochratoxin A in the presence of 1 or 5 mg rubratoxin B/kg is shown in Table 2. When 24-hr-old littermates were exposed simultaneously to a constant dosage of rubratoxin B and different doses of ochratoxin A, a decrease in the LD_{50} for ochratoxin A was observed. The LD_{50} value was reduced from 3·9 mg/kg for ochratoxin A alone to 0·24 and 2·5 mg/kg for ochratoxin in the presence of 5·0 and 1·0 mg rubratoxin B/kg, respectively. The value of 0·24 mg/kg for the LD_{50} of ochratoxin A in the presence of 5·0 mg rubratoxin B/kg was 16 times below the LD_{50} in the absence of rubratoxin B, representing a 16-fold increase in ochratoxin toxicity. When various doses of rubratoxin were given in the presence of 2 mg ochratoxin/kg, there was a 4·5-fold decrease in the rubratoxin LD_{50} compared with that for rubratoxin alone (Table 2).

The effect on weight gain of administering a constant dose of rubratoxin B (5 mg/kg) with different doses of ochratoxin A (0·1, 0·25, 0·5 or 1·0 mg/kg) is presented in Fig. 2. A significant decrease in weight gain was seen in pups treated with 5·0 mg rubratoxin B/kg plus a dose of 0·5 mg ochratoxin A/kg or more, but not when the ochratoxin A dose was 0·25 mg/kg or less. All pups died when the dose of ochratoxin A was 1 mg/kg or higher. Although the data are not included in this figure, all pups given 5·0 mg rubratoxin B/kg and 2·5 mg ochratoxin A/kg died within 6 days. Again an interactive dose response to the combined mycotoxins was seen in respect of weight gain.

Table 2. *Combination oral LD_{50} values for ochratoxin A and rubratoxin B in neonatal rats*

Dose levels (mg/kg)*		
Rubratoxin B	Ochratoxin A	LD_{50}(mg/kg)†
5·0	0–2·5	0·24 (0·19–0·36) (a)
1·0	0–3·0	2·50 (2·31–2·64) (a)
0–5·0	2·0	1·40 (1·34–1·46) (b)

*Animals received a constant dose of one mycotoxin 24 hr after birth and at the same time varying dose levels of a second toxin were administered.

†The LD_{50} values for ochratoxin A (a) and rubratoxin B (b) in the presence of a constant concentration of the second mycotoxin were calculated after 7 days. The values represent data from a minimum of ten animals.

In the multiple-treatment experiments, 24-hr littermates were divided into control and treated groups. Rubratoxin B (5 mg/kg) in corn oil was administered by stomach tube to the treated group and corn oil was administered to control animals. Fourteen days after the primary dosing, the treated and control groups were each divided into two subgroups. Pups in one subgroup of each of the original groups were given 4 mg aflatoxin B_1/kg, while pups in the other subgroup were given corn oil alone, so that the pups in the four groups received either corn oil on days 1 and 15, corn oil on day 1 and aflatoxin on day 15, rubratoxin B on day 1 and corn oil on day 15 or rubratoxin on day 1 and aflatoxin on day 15. There was a significant reduction of 30% in the average daily weight gain between rubratoxin-treated animals and control animals over the first 14 days of the experiment. In animals given rubratoxin (5 mg/kg) followed by aflatoxin B_1 (4 mg/kg) on day 15, average daily weight gains for the 30-day observation period were reduced in comparison with all the other three groups (Table 3). Rubratoxin-treated animals showed a 30% decrease in daily weight gain compared with control animals during the first 14 days after initial treatment. After treatment with aflatoxin B_1 on day 15, animals treated initially with rubratoxin B showed an 18% decrease in daily weight gain compared with the rubratoxin-control animals and a 30% decrease compared with animals treated only with corn oil. The decrease in daily weight gain of animals treated with rubratoxin followed by aflatoxin appeared to be mediated by both agents rather than by either agent alone. Lowering the doses of rubratoxin and aflatoxin by 50% gave similar results; however, the decreases in weight gains were only about half of the values obtained with the higher dose levels (Table 4). In the

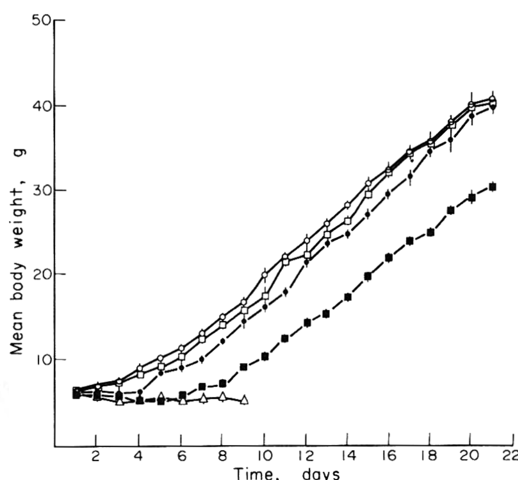

Fig. 2. Effect on weight gain of the simultaneous oral administration of 5·0 mg rubratoxin B/kg together with ochratoxin A in a dose of 0 (control; ○), 0·10 (□), 0·25 (●), 0·50 (■) or 1·00 (△) mg/kg. Values are the means ± SEM for a minimum of ten animals.

Table 3. *Effects on body weight of administering orally to rats rubratoxin B (5 mg/kg) 24 hr after birth followed by aflatoxin B_1 (4 mg/kg) on day 15*

Treatment		Mean daily weight gain (g)* at days		Mean body weight (g)* at day 30
At 24 hr	On day 15	1–14	15–16	
CO	—	1·93 ± 0·12		
Rubratoxin	—	1·35 ± 0·27[a]		
CO	CO		4·30 ± 0·16	93·75 ± 4·1
CO	Aflatoxin		4·26 ± 0·31	96·10 ± 4·9
Rubratoxin	CO		3·65 ± 0·26[a]	81·20 ± 6·2[ab]
Rubratoxin	Aflatoxin		3·00 ± 0·36[a]	66·90 ± 5·9[abc]

CO = Corn oil (control)

*Values are means ± SEM for a minimum of ten rats and those marked with superscripts differ significantly ($P < 0.05$ by Student's t test) from the corresponding value for the ([a]) CO or CO–CO control group, ([b]) CO–aflatoxin group and/or ([c]) rubratoxin–CO group.

reverse experiment, in which aflatoxin B_1 (0·4 mg/kg) was followed by rubratoxin B (40 mg/kg), no synergistic response was demonstrated (data not presented).

In a similar experiment, oral treatment with 2 mg ochratoxin A/kg at 24 hr did not enhance the effect of 40 mg rubratoxin/kg given on day 15. Animals treated initially with ochratoxin showed approximately the same percentage decrease in daily weight gains as animals subsequently treated with either rubratoxin or corn oil (data not presented).

No significant differences between treated and control groups were observed in liver-to-body weight ratios in any of the experiments.

DISCUSSION

These experiments demonstrated that neonatal rats were more susceptible to aflatoxin B_1, ochratoxin A and rubratoxin B than were adult animals. The adult-to-neonate LD_{50} ratios were 9·2, 6·5 and 49·2 for aflatoxin B_1, ochratoxin A and rubratoxin B, respectively. Lethality and effects on weight gain were additive or interactive in the case of aflatoxin B_1 and rubratoxin B but not in the case of ochratoxin A and rubratoxin B. It appears that initial exposure to rubratoxin enhances the susceptibility of young animals to subsequent exposure to aflatoxin, whereas the reverse did not reflect a sensitization by aflatoxin to the toxic effects of rubratoxin. A similar synergistic effect has been demonstrated in weanling rats after simultaneous dosing with aflatoxin B_1 and rubratoxin B when single doses of the mycotoxins showed no effect (Wogan *et al.* 1971). Experiments described here also showed that a delayed response was associated with sequential dosing of rubratoxin at 5 mg/kg on day 1 followed by 4 mg aflatoxin/kg on day 15. Neither of the other combinations used showed this delayed effect.

Since most mycotoxin-producing fungi have essentially the same environmental requirements for toxin production and infect many of the same substrates (Hayes & Wilson, 1968), multiple exposure and/or delayed exposure to mycotoxins should not be unexpected. Interactive and/or delayed effects demonstrated by the data presented in this paper reflect another toxicity problem that can occur when more than one toxin-producing mould contaminates the same substrate. Field exposures to aflatoxin and ochratoxin have been observed recently in chickens (P. B. Hamilton, personal communication 1976). Exposure of young animals to such active agents in the environment could make the adult more susceptible to similar agents or to other environmental toxicants such as pesticides (Farb, Sanderson, Moore & Hayes,

Table 4. *Effects on body weight of administering orally to rats rubratoxin B (2·5 mg/kg) 24 hr after birth followed by aflatoxin B_1 (2 mg/kg) on day 15*

Treatment		Mean daily weight gain (g)* at days		Mean body weight (g)* at day 30
At 24 hr	On day 15	1–14	15–16	
CO	—	1·95 ± 0·11		
Rubratoxin	—	1·57 ± 0·17[a]		
CO	CO		3·61 ± 0·15	87·0 ± 5·6
CO	Aflatoxin		3·44 ± 0·29	86·3 ± 6·1
Rubratoxin	CO		3·18 ± 0·22[a]	80·0 ± 8·1
Rubratoxin	Aflatoxin		2·88 ± 0·31[a]	70·6 ± 6·4[a]

CO = Corn oil (control)

*Values are means ± SEM for a minimum of ten rats and those marked with a superscript ([a]) differ significantly ($P < 0.05$ by Student's t test) from the corresponding value for the CO or CO–CO control group.

1973). Because of the ubiquity of mycotoxins, such studies are important in detecting potential health hazards to man and in establishing control measures that will remove such agents from food chains.

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CITRININ MYCOTOXICOSIS IN THE MOUSE

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Abstract—Citrinin was mixed in a purified diet for feeding to mice or was dissolved in sodium hydroxide (NaOH) or in equal parts of dimethylsulphoxide (DMSO) and 50% ethanol (EtOH) for administration by the ip, sc and oral routes. The single-dose LD_{50} values determined in male mice at 72 hr for citrinin dissolved in DMSO–50% EtOH were 58, 73 and 112 mg/kg for the ip, sc and oral routes, respectively. The ip and oral LD_{50} values in male mice given citrinin dissolved in 0·5 N-NaOH were 80 and 105 mg/kg, respectively. The ip LD_{50} values for female mice given citrinin dissolved in DMSO–50% EtOH and in 0·5 N-NaOH were 62 and 87 mg/kg, respectively. The death rate and pathological changes produced by a single dose of citrinin were not made more severe by multiple doses. Lesions were confined to the kidney and included renal tubular dilatation, the presence of protein and protein casts within the lumen of the renal tubules, slight to moderate necrosis of the renal cortical tubular epithelium and a slight increase in mitotic activity in the medullary tubular epithelium. Citrinin dissolved in DMSO and 50% EtOH retained its biological activity when stored in a refrigerator for 1 month, but activity was lost when the citrinin solution was heated to 90°C for 10 min.

INTRODUCTION

Citrinin is a secondary metabolite produced by a number of fungal species belonging to the genera Penicillium and Aspergillus (Hetherington & Raistrick, 1931; Korzybski, Kowszyk-Gindefer & Kurylowicz, 1965; Pollock, 1947; Timonin & Rouatt, 1944a). It was produced by fungi isolated from feed grains in Canada (Scott, van Walbeek, Kennedy & Anyeti, 1972) and in Denmark (Friis, Hasselagar & Krogh, 1969; Krogh, Hald & Pedersen, 1973). Citrinin in combination with ochratoxin A has been incriminated in natural outbreaks of mould nephrosis in Danish swine (Krogh *et al.* 1973), but there have been no reports linking citrinin contamination with field outbreaks of mycotoxic disease in animals outside Denmark.

The acute toxicity of citrinin dissolved in sodium hydroxide, neutralized with hydrochloric acid and given by various routes was determined for mice, rats, guinea-pigs and rabbits (Ambrose & DeEds, 1945 & 1946). Renal lesions in the guinea-pig and rabbit consisted primarily of swelling, necrosis and desquamation of the epithelium of the proximal convoluted tubules (Ambrose & DeEds, 1946). Clinical signs following ip administration of citrinin (20–40 mg/kg body weight) to young beagle dogs were vomiting and immediate defaecation and urination (Carlton, Sansing & Szczech, 1974). No gross lesions were found except intestinal intussusception, and histological changes were confined to the kidneys. Necrosis of individual cells and of small groups of epithelial cells were seen most often in the straight segments and in the distal convoluted tubules. Similar lesions were seen in the kidneys of citrinin-fed swine and rats (Krogh, Hasselager & Friis, 1970).

Data available on the toxicity of citrinin in other species are limited. This report describes studies of the acute toxicity of citrinin in the mouse, the mycotoxin being given by various routes and in two different solvents.

EXPERIMENTAL

Material. Citrinin, prepared as previously described by Carlton *et al.* (1974), was mixed in a purified diet (Mills & Murray, 1960) for feeding or dissolved in 0·5 N-sodium hydroxide or in dimethylsulphoxide–50% ethanol (1:1, v/v; DMSO–50% EtOH) for administration by the oral, sc or ip route.

Animals. Weanling Swiss mice of the ICR strain, weighing 10–15 g, were purchased from Harlan Industries, Cumberland, Ind., and housed in shoebox plastics cages. Groups were maintained in laboratory animal rooms at temperatures between 22 and 26°C. Feed and water were supplied *ad lib.*

Collection of tissues. Mice found moribund during the experiments and the survivors killed at termination were autopsied. The right kidney and the liver, heart, and spleen were fixed in neutral buffered 10% formalin. A midsagittal section of the left kidney was placed in Zenker's acetic acid fixative. Fixed tissues were dehydrated, embedded in paraffin, sectioned at 4 and 6 μm and stained with Masson's trichrome stain or haematoxylin and eosin for histopathological examination.

Design of experiments

Trial I. Citrinin prepared as a premix with cerulose or dissolved in ethanol at a concentration of 5 mg/ml was used for feeding studies. The citrinin was combined with a semi-purified diet (Mills & Murray,

1960) and fed for 2 wk at dietary concentrations of 50–2000 ppm. Control groups were fed either the basic diet or this diet with addition of an appropriate quantity of ethanol.

Trial II. The toxicity of the DMSO–EtOH solvent used in many of the following experiments was studied. The solvent consisted of a stock solution of DMSO mixed with equal parts (v/v) of 50 or 70% EtOH. Following oral, ip or sc administration, the single-dose LD_{50} for the solvent and the upper and lower confidence limits ($P = 0{\cdot}05$) were calculated (Litchfield & Wilcoxon, 1949). Mice were killed 72 hr after injection for post-mortem examination and the collection of tissues for histopathological examination.

Trial III. The single-dose toxicity of citrinin in DMSO–50% EtOH or in neutralized 0·5 N-NaOH was studied. Citrinin was mixed with DMSO–EtOH at various concentrations so that the dosage of solvent was generally 7 ml/kg body weight when given by the ip and sc routes. The amount of DMSO–EtOH solvent for citrinin administration by the oral route was 11 ml/kg for most of the mice. Citrinin was dissolved in 0·5 N-NaOH (10 mg/ml) by the method described by Timonin & Rouatt (1944b) and later used by Ambrose & DeEds (1945 & 1946). Appropriate quantities of the citrinin–NaOH solution were administered to obtain the designated dose of citrinin. The LD_{50} values for citrinin in these two solvents administered by the ip, sc and oral routes were calculated as in Trial II. Survivors were killed 72 hr after injection for autopsy and tissues were collected for histopathological examination.

Trials IV & V. These were conducted to determine the stability of citrinin in the DMSO–50% EtOH solvent. In Trial IV, ten male mice were given single ip injections of citrinin in a dose of 73 mg/kg body weight. The remaining citrinin solution was stored for 1 month in the dark at 4°C. This solution was then injected into a similar group of mice and the death rates in the groups given the fresh and stored solutions were compared. The heat-stability of citrinin dissolved in the DMSO–50% EtOH solvent was studied in Trial V. A freshly prepared solution of citrinin was administered to ten male mice in a single ip dose of 73 mg/kg. In experiment A, the remaining solution was heated with stirring for 10 min at 90°C. It was then administered to mice in the same dose and the number of deaths in the two groups was compared. Experiment B was similar except that the citrinin solution was heated to 83°C for 8 min.

Trial VI. The effects of multiple doses of citrinin dissolved in DMSO–50% EtOH (1:1, v/v) and injected in a volume of 5–8 ml/kg were studied in mice given sc or ip injections of citrinin daily or every other day for 1 or 2 wk. The death rate in these mice and the pathological changes in mice killed at 7 and 14 days were compared with those in mice given single doses of citrinin at similar levels.

Trial VII. The sequential development of renal lesions over a 7-day period was studied in mice given daily ip injections of 55 mg citrinin/kg body weight dissolved in DMSO–50% EtOH. The concentration of citrinin in the solvent was 11 mg/ml. Groups of ten mice were killed on injection days 3 and 5 and the remaining 11 mice were killed on day 7. Control mice received the solvent for similar periods of time. Renal lesions were compared in the treated and control mice killed at a given time. Mice that died were not examined histopathologically.

Trial VIII. This study was made to evaluate the earliest changes in the kidneys of mice given citrinin. The mycotoxin, dissolved in 0·5 N-NaOH and neutralized to pH 6·5–7·0 with HCl was given to ten mice in a single oral dose of 130 mg/kg body weight. One mouse was killed every 2 hr from 2 to 12 hr after treatment. The remaining mice were killed at 24 hr. Renal lesions in the mice killed at the various times were compared.

RESULTS

Trial I

Clinically, treated mice could not be distinguished from control mice. Average body-weight gains were reduced by dietary concentrations of citrinin at and above 500 ppm, but the number of deaths was increased only in the group fed citrinin at a concentration of 2000 ppm (Table 1). No gross changes were present at autopsy. There were no renal lesions. Disseminated focal necrosis and infiltration of mononuclear leucocytes and a few polymorphonuclear leucocytes were observed in the livers of several of the test mice, and rod-shaped gram-positive bacteria were found within many of these lesions. Temporary problems with the environmental control systems of the room were believed to have been responsible for the development of active disease apparently caused by *Corynebacterium kutscheri* (Fauve, Pierce-Chase and Dubos, 1964). Feeding was discontinued after 14 days because of poor control over the intake of citrinin and a lack of information on the stability of citrinin in the feed.

Trial II

Mice given the solvents DMSO–50% EtOH and DMSO–70% EtOH either by the ip or oral route were hyperactive for 5–10 min, but soon became markedly

Table 1. *Body weight gains and death rates in mice fed various dietary concentrations of citrinin (Trial I)*

Citrinin concn (ppm)	Weight (g) at day			No. of deaths†
	0*	7	14	
Experiment A				
0 (control)	13	16	15	1/10
0 (control‡)	12	15	14	2/10
50	12	16	14	2/10
100	13	15	14	3/10
250‡	12	15	14	0/10
Experiment B				
0 (control)	13	16	19	1/15
500	13	14	16	0/15
1000	13	13	14	2/15
1500	14	12	14	2/10
2000	10	—§	—§	6/10§

*Initial body weight.
†No. dead at day 14/initial no.
‡With alcohol.
§Terminated at day 5.

depressed and remained so for 1–2 hr. These changes were less marked when the solvent was given sc. The LD_{50} values for DMSO–50% EtOH were 15, 18 and 23 ml/kg for the ip, sc and oral routes, respectively (Table 2). Diffuse fibrinous peritonitis occurred in 16/50 mice injected ip with DMSO–70% EtOH, while mild focal fibrinous peritonitis was occasionally observed in mice injected with DMSO–50% EtOH. In the kidneys, the tubular epithelium of the cortex and distal convoluted tubules was necrotic in four mice treated with DMSO–50% EtOH ip or sc in doses of 15–19 ml/kg body weight.

Trial III

Mice given citrinin ip or orally in DMSO–50% EtOH or in 0·5 N-NaOH were hyperactive for 5–10 min, but soon became markedly depressed and remained so for 2–4 hr. Similar, but less severe, behavioural changes were observed when citrinin was administered by the sc route. Lachrymation was increased in mice given citrinin, as indicated by a small quantity of clear fluid on the eyelids, and was proportional to the dose administered. Citrinin was most toxic by the ip route, less so by the sc route and least toxic when given by the oral route (Table 3). The LD_{50} values varied from 40 to 112 mg/kg depending on the solvent and route of administration. The LD_{50} was slightly higher when 0·5 N-NaOH was the solvent for ip administration than when DMSO–50% EtOH was used. This difference was not observed when citrinin was given orally in these two solvents. No sex difference in susceptibility to citrinin was found. Increasing the amount of the DMSO–50% EtOH solvent injected reduced the LD_{50} for citrinin dissolved in this solvent and administered by the sc route.

Table 2. *Single-dose toxicity of DMSO–EtOH mixtures administered by various routes to male mice (Trial II)*

Solvent*	Route	No. of mice	LD_{50} (ml/kg)	Range ($P = 0·05$)
DMSO–70% EtOH	ip	40	9	8–11
DMSO–50% EtOH	ip	124	15	14–16
	sc	100	18	17–19
	oral	60	23	22–24

DMSO = Dimethylsulphoxide EtOH = Ethanol
*Equal parts (v/v) of DMSO and the given concn of EtOH.

Gross alterations attributable to citrinin were not observed. Significant histological changes were limited to the kidneys and included tubular dilatation, the presence of protein and protein casts within the tubules, necrosis of the tubular epithelium, and an increase in mitotic activity in the medullary tubular epithelium (Table 4). Renal lesions were not observed in any of the 164 control mice examined.

A positive correlation existed between the dose of citrinin and the frequency and severity of the renal lesions. Slight to moderate renal tubular dilatation most often involved the cortical tubules, but occasionally affected the tubules of the renal medulla (Fig. 1). Protein and protein casts were present in the tubular lumina in both the cortex and medulla, but were most frequent in the lumina of the tubules of the inner zone of the medulla. Protein and protein casts in trichrome-stained sections were seen as a pale blue, pink or intense red homogeneous material which filled or partially filled individual renal tubules (Fig. 2). Necrosis of individual cells or small groups of renal tubular epithelial cells was most extensive in the straight segments of the renal cortical tubules (Fig. 3). Necrotic cells were sometimes observed in the convoluted portions of the distal tubules and were seen occasionally in medullary tubules. Necrotic cells were rounded, often detached from the basement membrane and had pyknotic nuclei, and the cytoplasm of these cells was either vacuolated or very dense and in the latter case stained dark red with the trichrome stain. Desquamated cells were either free within the tubular lumina or were mixed with proteinaceous material filling these lumina. Basement membranes devoid of tubular epithelium were seen occasionally. The number of mitotic figures in the renal cortical tubules of test mice was no greater than the number counted in control mice. The mitotic index of the medullary renal tubular epithelium was significantly higher ($P = 0·05$) in mice treated with citrinin than in control mice (Table 4).

Trial IV

The biological activity of citrinin dissolved in DMSO–50% EtOH was not reduced by storage for 1 month in the dark at 4°C. The death rate among mice given the freshly prepared citrinin solution was

Table 3. *Single-dose toxicity of citrinin dissolved in various solvents and administered to mice by various routes (Trial III)*

Route of administration	Solvent	Experiment	Sex	Amount of solvent (ml/kg body weight)	No. of mice	LD_{50} (mg/kg body weight)	Range ($P = 0·05$)
Intraperitoneal	DMSO–50% EtOH	A	M	7	290	58	55–61
		B	F	7	120	62	58–66
	0·5 N-NaOH	C	M	6–11	110	80	75–86
		D	F	6–12	160	87	80–95
Subcutaneous	DMSO–50% EtOH	E	M	12	50	40	34–47
		F	M	7	230	73	71–75
Oral	DMSO–50% EtOH	G	M	7,10,11	110	112	101–124
	0·5 N-NaOH	H	M	11–16	160	105	98–112

DMSO = Dimethylsulphoxide EtOH = Ethanol

Table 4. *Renal lesions in mice given a single toxic dose of citrinin (Trial III)*

Route of adminis-tration	Solvent	Experiment	No. of mice examined	No. of mice showing evidence of											
				Tubular dilatation			Protein in tubules			Tubular necrosis			Increase in mitotic index of medullary tubules		
				S	M	Mo	S	M	Mo	S	M	Mo	S	M	Mo
Intraperitoneal	DMSO–50% EtOH	A	139	5	2	0	7	6	0	1	0	0	0	1	0
		B	59	3	0	1	9	1	0	2	2	2	3	0	0
	0·5 N-NaOH	C	79	6	1	0	9	4	1	11	1	0	10	1	1
		D	89	8	0	1	16	2	3	12	5	0	7	0	0
Subcutaneous	DMSO–50% EtOH	E	22	1	2	0	1	0	2	0	3	0	0	0	0
		F	93	7	2	3	26	4	4	16	2	3	5	0	0
Oral	DMSO–50% EtOH	G	61	7	1	0	12	8	7	7	5	6	11	1	0
	0·5 N-NaOH	H	55	20	10	6	13	10	14	8	14	12	15	0	0

S = Slight M = Mild Mo = Moderate DMSO = Dimethylsulphoxide EtOH = Ethanol

80% and in mice given this same solution after storage for 1 month the rate was 90%.

Trial V

The biological activity of the citrinin solution was reduced by heating. The death rate was 80% in mice given the freshly prepared citrinin solution but no deaths occurred in the group of mice given citrinin solution heated at 90°C for 10 min. In mice treated with citrinin solution heated to 83°C for 8 min, 50% died.

Trial VI

The number of deaths (Table 5) and the pathological changes produced by multiple sc and ip injections of citrinin did not differ significantly from those produced by a single injection of similar doses (Table 3). Oedema and induration developed at the sites of multiple sc injections. Renal lesions were found in only two of 30 mice given multiple sc injections of citrinin. These two mice received the highest doses (50 and 65 mg/kg) of citrinin. The lesions consisted of mild renal tubular dilatation, protein and protein casts in the tubular lumina and necrosis of a few individual renal tubular epithelial cells.

Multiple ip injections of citrinin reduced slightly the weight gains of the mice compared with those of control mice receiving only the solvent. The daily ip injection of solvent at the dose level (7 ml/kg) previously used for the single ip injection of citrinin resulted in fibrinous peritonitis, which was more severe when citrinin was administered along with the solvent. Of 44 mice treated with citrinin and examined histopathologically, four had renal lesions. Renal lesions produced by multiple ip injections of citrinin were similar to those produced by a single ip injection of the same dose of citrinin. A few individual necrotic renal cortical tubular epithelial cells were present in one mouse given multiple injections of citrinin at a dose of 40 mg/kg. Slight to moderate renal tubular dilatation, protein and protein cast formation and individual cell necrosis was observed in three mice given multiple ip injections of citrinin in doses of 50 mg/kg during wk 1 and 60 mg/kg during wk 2.

Trial VII

The incidence of renal lesions was not increased by multiple injections of citrinin and the changes in affected mice were not more severe. Mild renal tubular dilatation and necrosis of individual renal tubular epithelial cells were observed in one of ten mice killed after three daily ip injections of citrinin in doses of 55 mg/kg. Moderate renal tubular dilatation, mild protein and protein-cast formation in the tubular lumina and moderate necrosis of the renal tubular epithelium were present in one of ten mice killed after five daily ip injections of citrinin. Renal lesions were not observed in any of 11 mice killed after seven daily injections of citrinin.

Trial VIII

Mice were killed at 2-hr intervals after administration of a single oral dose of 130 mg citrinin/kg. Renal lesions were not seen in mice killed 2–6 hr after administration, but mild renal tubular dilatation was seen in a mouse killed at 10 hr. A few necrotic individual renal tubular epithelial cells were observed in mice killed after 8–12 hr and in one of the two mice killed 24 hr after citrinin administration. Necrosis of the renal cortical tubular epithelium was moderate in the second mouse killed at 24 hr.

DISCUSSION

Mice were less susceptible to the toxic effects of citrinin than were guinea-pigs (Ambrose & DeEds, 1945; Thacker, Carlton & Sansing, 1975) and beagle dogs (Carlton *et al.* 1974; Kitchen, Carlton & Sansing, 1975), as the several LD_{50} values determined for the mouse were greater than those reported for these other species. The LD_{50} values of citrinin for mice by sc and ip administration were similar to those reported for rats (Ambrose & DeEds, 1945), but were approximately twice those for the 14-day LD_{50} reported for mice by Ambrose & DeEds (1946). In Trial VI, multiple doses of citrinin at 25–75 mg/kg given over a 14-day period did not result in a greater proportion of deaths than was observed in Trial III

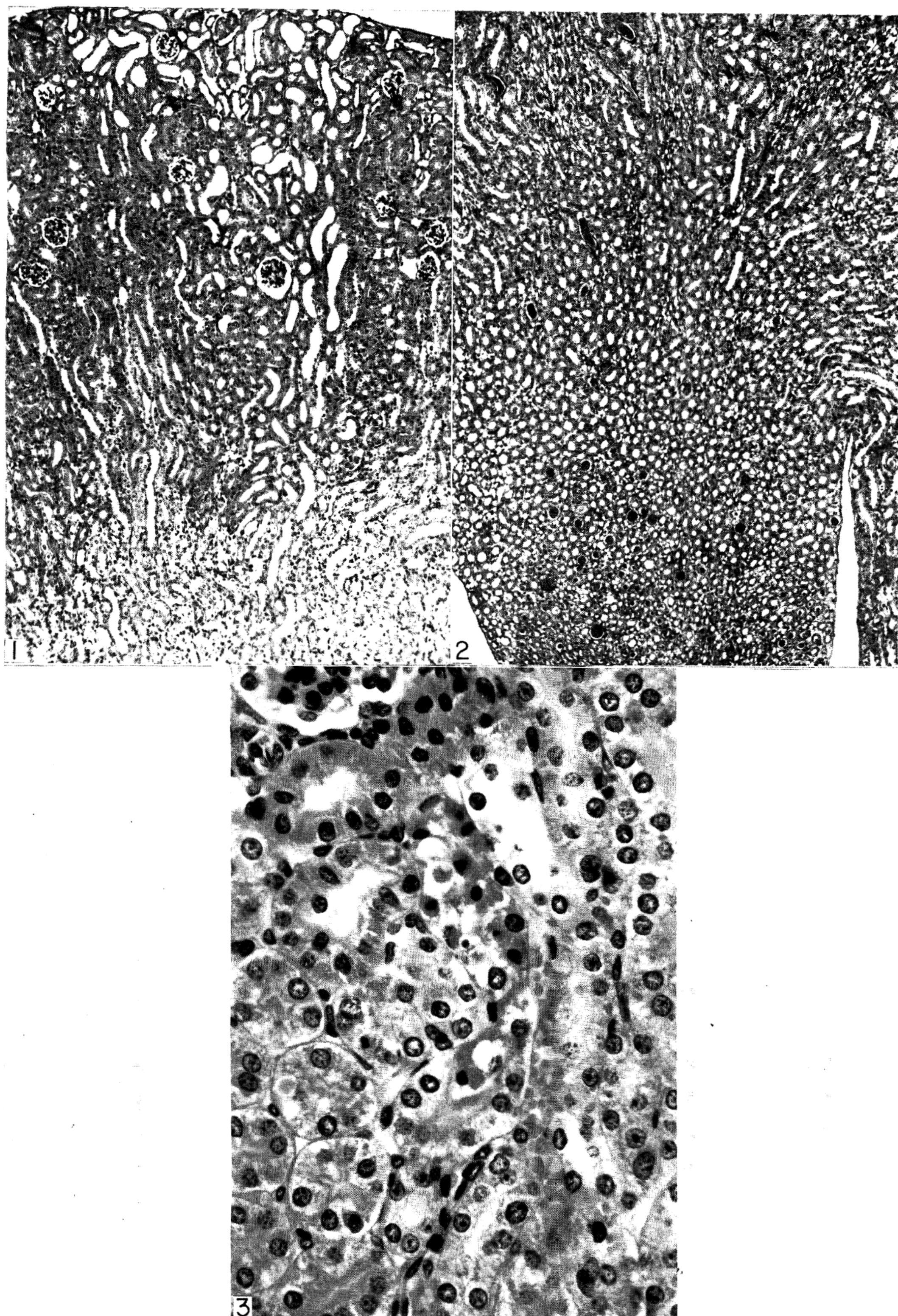

Fig. 1. Mild renal tubular dilatation in a mouse given a single ip dose of 70 mg citrinin/kg (Trial III). Haematoxylin and eosin × 63.

Fig. 2. Protein casts in the lumina of renal medullary tubules in a mouse given a single dose of 120 mg citrinin/kg (Trial III). Trichrome × 63.

Fig. 3. Necrosis of proximal renal tubular epithelium in mouse killed 24 hr after a single oral dose of 130 mg citrinin/kg (Trial VIII), showing pyknotic nuclei in several tubular cells (centre) and cytoplasmic and nuclear changes in individual cells of two tubules (left). Haematoxylin and eosin × 400.

Table 5. *Incidence of deaths in male mice following sc or ip injections of citrinin every day or every other day (Trial VI)*

Route of administration	Dose of solvent (ml/kg body weight) in wk 1	Dose of solvent (ml/kg body weight) in wk 2	Wk 1 Frequency of injection	Wk 1 Dose of citrinin (mg/kg body weight)	Wk 2 Frequency of injection	Wk 2 Dose of citrinin (mg/kg body weight)	No. of deaths* by day 7	No. of deaths* by day 14
Subcutaneous	7	–	ED	0†	—	—	0/10	—
	7	7	EOD	0†	EOD	0†	0/10	0/10
	7	–	ED	35	—	—	2/10	—
	7	7	EOD	35	EOD	35	0/10	0/10
	5	7	EOD	50	ED	65	0/10	2/10
Intraperitoneal	7	–	ED	0†	—	—	1/10	—
	7	7	EOD	0†	EOD	0†	0/10	1/10
	7	–	EOD	25	—	—	0/5	—
	7	–	ED	25	—	—	7/15	—
	7	7	EOD	25	EOD	25	1/10	1/10
	5	5	EOD	40	ED	40	3/10	5/10
	5	5	EOD	40	ED	50	1/10	4/10
	5	5	ED	50	ED	50	5/15	10/15
	5	6	ED	50	ED	60	2/10	4/10
	5	6	EOD	50	ED	60	2/10	2/10
	5	8	EOD	50	ED	75	0/5	5/5

ED = Injection every day EOD = Injection every other day

*Total no. dead at day 7 or 14/initial no.

†Control group.

with single doses of 25–75 mg/kg. This indicates that the difference between our results and those of Ambrose & DeEds (1946) was not due to the longer experimental period used by the latter investigators.

Neither can differences in death rates be ascribed to the solvent used, as the LD_{50} obtained by us for ip administration of citrinin dissolved in NaOH and neutralized with HCl, as described by Timonin and Rowatt (1944b) and used by Ambrose & DeEds (1945 & 1946), was greater than that for citrinin dissolved in DMSO–50% EtOH and administered by the same route. Others have described differences in LD_{50} values depending on solvent. Wogan, Edwards & Newberne (1971) observed that the LD_{50} of rubratoxin B for mice and cats was 5–10 times greater when propylene glycol was used as the solvent than when the toxin was dissolved in DMSO. No differences were observed in the LD_{50} values when these two vehicles were used for the administration of rubratoxin B to rats.

The route of administration can alter considerably the LD_{50} of a toxin, as was found for citrinin. The LD_{50} for orally administered rubratoxin B was over 1000 times that of the LD_{50} obtained for the ip route (Wogan *et al.* 1971). Purchase & van der Watt (1969) reported that the oral LD_{50} of sterigmatocystin for rats was twice that of the ip LD_{50}. Wogan *et al.* (1971) suggested that the acidic pH of the stomach was at least partially responsible for the decreased toxicity of rubratoxin B administered orally. The reduced toxicity of citrinin when given by the oral route may have been due to precipitation of the mycotoxin in the acid medium (Hetherington & Raistrick, 1931) leading to a reduction in its absorption. Purchase & van der Watt (1969) suggested that precipitation in the stomach of rats was responsible for some loss of toxicity of sterigmatocystin administered orally.

The renal lesions induced in mice by citrinin were less severe than those described in dogs (Carlton *et al.* 1974; Kitchen *et al.* 1975) and guinea-pigs (Ambrose & DeEds, 1946; Thacker *et al.* 1975). The distribution of the renal lesions also varied among species, as the straight segments of the proximal and distal tubules were most severely affected in the mouse and dog (Carlton *et al.* 1974), while in the rat (Krogh *et al.* 1970) and guinea-pig and rabbit (Ambrose & DeEds, 1946), the epithelial cells of the proximal convoluted tubules were most severely affected.

Intestinal intussusception, apparently a parasympathomimetic response to citrinin, was observed in dogs (Carlton *et al.* 1974; Kitchen *et al.* 1975) and guinea-pigs (Thacker *et al.* 1975) but was not observed in mice. The absence of renal lesions in many moribund mice after citrinin administration also suggested that citrinin profoundly affected organ systems other than the kidney, but the cause of death in mice without detectable renal lesions was not determined.

The absence of any effect on death rate or lesion severity after multiple injections of citrinin was unexpected. Because the mechanism of toxicity of citrinin remains essentially unknown and biochemical pathways have not been explored, no satisfactory explanation can be offered. It is possible that a citrinin-sensitive cell population was destroyed by the first dose and the surviving cells were not sensitive to citrinin toxicity. It is also possible that enzyme systems re-

sponsible for the detoxication of citrinin were induced and subsequent doses of citrinin were more rapidly metabolized. Rats were able to withstand multiple doses of rubratoxin over a prolonged period and Wogan *et al.* (1971) suggested that the toxin was effectively detoxified or excreted.

The biological activity of citrinin dissolved in DMSO–50% EtOH remained high when the solution was stored in a dark refrigerator for as long as 1 month. It was important to keep the citrinin solution in the dark, as it turned red within 1–2 wk when kept in the light at room temperature. Citrinin in NaOH solution also acquired a red discoloration within 1 wk when stored either at room temperature or at 4°C. Ambrose & DeEds (1946) suggested that the red colour was due to irreversible oxidation of citrinin. The toxicity of the discoloured citrinin solution was not tested, but it was assumed that the toxicity was reduced. A citrinin solution in NaOH should not be prepared more than a few minutes before use.

Although citrinin can be dissolved more easily in DMSO–50% EtOH when heated, the heating, if not controlled, can result in a reduction in activity, as demonstrated in this study. Decomposition of citrinin as a result of heating was also reported by Kawashiro, Tanabe, Takeuchi & Nishimura (1956) and by Neely & Ellis (1972). Citrinin in hexane and ethanol underwent decomposition at temperatures above 60°C. Timonin (1942) and Timonin & Rouatt (1944b) reported that autoclaving serial dilutions of citrinin in broth for 20 and 30 min at 15 lb/in^2 did not destroy the bacteriostatic activity of citrinin.

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EFFECTS OF VITAMIN A ON TOXICITY OF HEXACHLOROPHENE IN THE RAT

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Abstract—Male rats were given vitamin A acetate orally either daily in a dose of 0, 0·83 or 10·0 mg/kg or every third day at three times this dose, 2 wk before and continuing after daily administration of 50 or 75 mg hexachlorophene (HCP)/kg. At certain levels and regimens, vitamin A offered partial protection against HCP toxicity, in that it retarded the rate at which deaths occurred but not the eventual outcome. The highest level of vitamin A (30 mg/kg) given every third day for 2 wk did not alter significantly the single-dose LD_{50} of HCP. Vitamin A deficiency produced by omission of the vitamin from the diet considerably increased the toxicity of HCP after daily dosing at various levels. Cerebrospinal-fluid pressure in vitamin A-deficient control rats was 88 mm saline compared with 251 mm in HCP-treated normal rats. These quantitative differences suggest that these two factors exert independent and possibly additive effects rather than that vitamin A plays a primary role in the genesis of HCP neurotoxicity. Results do indicate, however, that the nutritional status of the animal affects its susceptibility to toxic halogenated hydrocarbons.

INTRODUCTION

Hexachlorophene (HCP) is a neurotoxic substance. Its effects upon the central nervous system include paralysis associated with oedema and spongy degeneration of the white matter of the brain and spinal cord (Kimbrough, 1971; Kimbrough & Gaines, 1971; Lockhart, 1972; Shuman, Leech & Alvard, 1973) and with the elevation of cerebrospinal-fluid pressure (CSFP) (Hanig, Krop, Morrison & Colson, 1973a & 1976; Hanig, Morrison & Krop, 1973b). Several factors point to the possibility that the vitamin A status of an animal may directly affect its susceptibility to HCP toxicity. For instance, it has been reported that both hypervitaminosis A (Marie & See, 1954) and hypovitaminosis A (Eleveth, Bolin & Goldsby, 1949; Hentges, Grummer, Phillips, Bohstedt & Sorensen, 1952; Lamming, Woollam & Millen, 1954; Millen & Dickson, 1957; Moore & Sykes, 1940 & 1941) raise the CSFP in mammals. The neurological signs of hypovitaminosis A, including hindlimb paralysis and convulsions, resemble the signs of HCP intoxication.

Various halogenated hydrocarbons that are capable of damaging the liver, such as bromobenzene, chlorinated naphthalenes, pentachlorophenol and certain insecticides, show enhanced toxicity in the vitamin A-deficient animal and their toxicity in the normal animal can be diminished by massive doses of vitamin A (Green, 1966). Although this cannot be taken as evidence for a direct role of vitamin A in the toxicity of these halogenated hydrocarbons, it suggested the desirability of investigating the role of this vitamin in both susceptibility to the neurotoxic effects of HCP and the development of a possible rational approach to the question of antagonism in the acute and chronic toxicity of HCP.

EXPERIMENTAL

Animals and maintenance. Male rats of the Osborne–Mendel strain from the FDA colony, weighing 100–200 g, were housed individually in a humidity-controlled animal room maintained at 22°C and were kept on a 12-hr light–dark cycle. They were given free access to standard Purina rat chow and water.

Vitamin-supplementation studies

Subacute experiment. As a pretreatment, vitamin A acetate dissolved in corn oil was administered by gavage (0·83 or 10 mg/kg) daily for 2 wk to groups of 15 rats or at three times this dose every third day for 2 wk to groups of six rats, two groups of control rats being given corn oil only. At the end of the pretreatment period, HCP in a 10% emulsion in corn oil containing 1% Tween was given by gavage in a dose of either 50 or 75 mg/kg daily for 45–66 days, and vitamin A administration was continued throughout the experiment. A limited number of control rats received only the vitamin at the high or low level, to establish the effect of the vitamin alone.

Acute experiment. In the acute LD_{50} experiment, 50 rats were given vitamin A (30 mg/kg) in corn oil by gavage every third day for 2 wk prior to administration of HCP, an equal number of controls being given a similar volume of corn oil. Both control and vitamin-treated rats were then divided into five subgroups each of ten rats and were given a single dose of HCP by gavage at a dose level of 75, 100, 150, 200 or 300 mg/kg. Vitamin A treatment was discontinued at this time and deaths during the following 14 days were recorded.

Vitamin-depletion studies

In deficiency studies, 40 weanling rats were fed a powdered diet with a normal content of all nutrients except that it was free of vitamin A and 40 additional weanlings were fed a control diet identical except for the inclusion of 10,000 IU vitamin A/kg. After 10 wk,

vitamin A deficiency was confirmed in the experimental rats by determining liver levels of vitamin A by the trifluoroacetic acid method of Neeld & Pearson (1963). This method was also used to check levels of vitamin A in the special diets and to determine whether changes in liver concentrations of vitamin A were occurring after HCP administration. Each group was divided into subgroups of ten rats, maintained on the same dietary regimen and dosed daily with HCP at one of four different levels (10, 25, 50 or 75 mg/kg).

The effects of vitamin deficiency, with or without HCP treatment, upon CSFP was the subject of a separate study using four groups of ten rats. The groups were given either a normal diet, a normal diet and two or three doses of HCP (50 mg/kg) by gavage, a vitamin A-deficient diet, or a vitamin A-deficient diet and HCP (50 mg/kg) by gavage. After 1 month, the CSFP of the 24 surviving rats was measured under barbiturate anaesthesia by a modification of the method of Hayes & Corey (1970), involving the insertion of a 27-gauge needle into the cisterna magna and recording from a Statham P-23 pressure transducer on a Grass Mode 7 polygraph. A three-way valve mounted on the pressure transducer was used to calibrate the polygraph to a full-scale deflection with a measured 100-mm column of saline. Analysis of variance was used to evaluate the statistical significance of the differences observed in CSFP between the various treatment groups.

RESULTS

Vitamin A supplementation

Attempts to antagonize the toxic effects of HCP with vitamin A met with a variable degree of success. Table 1 shows a comparison of the times by which 25, 50 or 100% of the rats had died in the groups given vitamin A every third day with daily doses of HCP. A death rate of 25% was reached in 6·6 days in the control group given 50 mg HCP/kg but no vitamin supplementation, but only after 30 days in the group given this HCP dose with 2·5 mg vitamin A/kg. At 12 days, 50% of the control group had died, but this figure was not reached in the vitamin-treated group during more than 42 days of observation. The animals treated with 2·5 mg vitamin A/kg thus appeared to have partial protection.

Table 1. *Periods in which 25, 50 and 100% of rats died when given 0–30 mg vitamin A/kg every third day and 50 or 75 mg HCP/kg daily by gavage*

Vitamin A dose (mg/kg)	HCP dose (mg/kg)	No. of days* required for death rate to reach 25%	50%	100%
0	50	6·6	12·0	>42·0
2·5	50	30·0	>42·0	>42·0
30·0	50	4·0	7·0	>42·0
0	75	3·4	4·5	7·0
2·5	75	2·5	4·0	5·0
30·0	75	5·5	7·0	19·0

HCP = Hexachlorophene
*Values are given for groups of six rats.

Table 2. *Periods in which 25, 50 and 100% of rats died when given 0–10 mg vitamin A/kg daily and 50 or 75 mg HCP/kg daily by gavage*

Vitamin A dose (mg/kg)	HCP dose (mg/kg)	No. of days* required for death rate to reach 25%	50%	100%
0	50	5·9	>49·0	>49·0
0·83	50	9·8	>49·0	>49·0
10·00	50	10·5	36·0	>49·0
0	75	3·5	4·6	30·0
0·83	75	3·5	5·5	>49·0
10·00	75	5·0	8·1	>49·0

HCP = Hexachlorophene
*Values are given for groups of 15 rats.

In contrast, rats treated with a higher level of vitamin A (30 mg/kg) seemed to have a slightly greater susceptibility to 50 mg HCP/kg, 4 and 7 days being needed to reach death rates of 25 and 50%, respectively, compared with 6·6 and 12 days for the control group. The lower dose of vitamin A had little effect in rats given 75 mg HCP/kg, 100% mortality being reached in the treated group 2 days earlier than in the control group. On the other hand, the higher vitamin dose appeared to offer some protection against 75 mg HCP/kg, with some of these rats surviving until day 19. No deaths occurred in the groups receiving either the low or high dose of vitamin A alone.

Table 2 shows the results of a similar experiment in which vitamin A was administered daily in doses equal to one-third of those given on every third day. In rats given 50 mg HCP/kg, both dose levels of vitamin delayed the attainment of a 25% death rate; however, in the group given the higher vitamin dose, 50% had died by day 36, whereas this situation was not reached in the control and low-vitamin groups during a period of more than 49 days. When HCP was given at 75 mg/kg, the low-vitamin regimen had little effect at the 25 and 50% mortality levels but appeared to prevent 100% mortality. With the higher daily dose of vitamin (10 mg/kg), results were similar to those in rats treated every third day, with apparently beneficial or protective effects at all mortality levels.

LD_{50} study

An LD_{50} study was performed to establish whether pretreatment with vitamin A had any protective effect against single massive doses of HCP. There was no significant difference between the LD_{50} values obtained in controls (180 mg/kg) and in rats treated with vitamin A (190 mg/kg), except that the response was more variable, the range of 19/20 confidence limits being 100–361 in the vitamin-pretreated group and 156–208 in the control group.

Vitamin A depletion

Rats given diets without vitamin A were shown by liver analysis to be deficient in the vitamin at the time of HCP administration. They were not debilitated by the deficiency at this time; although slight weight losses had occurred, no other signs were present.

Table 3. *Effect of vitamin A depletion on HCP toxicity in rats*

HCP dose* (mg/kg)	Diet†	No. of days‡ required for death rate to reach		
		25%	50%	100%
10·0	+A	>20·0	>20·0	>20·0
	−A	10·4	13·0	>20·0
25·0	+A	2·8	5·0	>20·0
	−A	3·4	3·6	7·0
50·0	+A	2·4	4·0	11·0
	−A	1·2	1·9	3·0
75·0	+A	2·6	3·5	4·0
	−A	2·2	3·0	3·5

HCP = Hexachlorophene
*Daily administration of HCP by gavage was started when the rats had been on the given diet for 10 wk.
†+A, diet containing 10,000 IU vitamin A/kg; −A, diet without vitamin A.
‡Values are given for groups of ten rats.

Table 3 shows the effects of vitamin A depletion on HCP toxicity. At the HCP dose level of 10 mg/kg, the group of rats fed the normal diet (+A) never reached 25% mortality, whereas in the group of vitamin-deficient rats (−A) 25% died in 10·4 days and 50% in 13 days. With doses of 25 mg HCP/kg, there was little difference between the groups in the times by which 25% of the rats had died, but in the −A group, 50% died in 3·6 days, compared to 5·0 days in the +A group. Deaths in the control group did not reach 100% during the 20-day observation period, but all the rats in the −A group died in 7·0 days. The effect of diet was clear with the HCP dose of 50 mg/kg, the three death rates being reached more rapidly in the −A group than in the +A group. At 75 mg/kg, diet had no apparent effect on either the rate of deaths or the final survival figures.

Effects of HCP and vitamin A deficiency on CSFP

The effects of HCP neurotoxicity and vitamin A deficiency upon CSFP were compared (Table 4). Rats kept on a vitamin A-deficient diet for 1 month had approximately double the CSFP value of untreated controls, whereas HCP-treated rats kept on a normal diet had CSFP values elevated to almost six times that of the controls. Measurements of CSFP in vitamin A-deficient rats given HCP were not obtained because most of the rats died so rapidly.

Table 4. *Effects of HCP and vitamin A deficiency on CSFP in the rat*

Group	CSFP* (mm saline)	No. of rats	*P*†
Control	40·0 ± 5·5	9	
Vitamin A-deficient	88·2 ± 15·4	10	<0·005
HCP-treated (50 mg/kg)	251·0 ± 52·9	5	<0.01

HCP = Hexachlorophene
CSFP = Cerebrospinal-fluid pressure
*Values are means ± SEM for the no. of rats stated.
†Analysis of variance; significance at $P < 0{\cdot}05$.

DISCUSSION

Experiments involving the pretreatment of rats with vitamin A as a protection against the toxic effects of HCP gave rather mixed effects. In several instances (Tables 1 and 2), low doses of vitamin A protected against low doses of HCP, whereas high doses of the vitamin enhanced the toxicity of low doses of HCP. In contrast, in other experiments high doses of vitamin A apparently decreased the toxicity of high doses (75 mg/kg) of HCP, but the lower dose of the vitamin in these instances was ineffective. Since both an excess and deficiency of vitamin A can result in elevation of CSFP (Marie & See, 1954; Moore & Sykes, 1940 & 1941) and since this elevation is one of the primary neurotoxic effects of HCP, it would seem that any interaction between these two compounds that results in either increased or attenuated toxicity is dependent upon the nutritional and physiological status of the individual at the time of HCP administration. Certain halogenated hydrocarbons are capable of acting, by a variety of mechanisms, as either vitamin A antagonists or antivitamins (Green, 1966; Leach & Lloyd, 1956). Numerous examples of vitamin A supplementation attenuating the toxic effects of halogenated hydrocarbons have been reported (Green, 1966).

Two possible primary sites of interaction between vitamin A and HCP are the liver and the brain. In the brain, the integrity of the apparatus for CSF absorption, comprising the endothelial cells of the arachnoid villi of most mammals or the torcular Herophili of the rat (Mann, Butler & Bass, 1975), is maintained or may be deranged by appropriate levels of vitamin A (Davson, 1967; Hayes, McCombs & Faherty, 1971; Witzel & Hunt, 1962), whereas the integrity of the myelin sheath is disrupted by HCP, due to splitting and demyelination (Kimbrough & Gaines, 1971; Lampert & Schochet, 1968; Towfighi, Gonatas & McCree, 1973). The liver contains considerable stores of vitamin A and is also an important site for the metabolism of HCP (Gandolfi, Nakaue & Buhler, 1974). The results from our vitamin supplementation experiments do not resolve the question of where the interaction of vitamin A and HCP occurs, if at all, or whether each compound acts individually, at different sites, on synergistic or antagonistic systems.

Our experiments in vitamin A-deficient animals indicate that in almost all instances the death rate is increased at various dose levels of HCP when the vitamin is absent. This increased susceptibility to HCP in the vitamin-deficient animal raises the question of the degree or extent to which each of the challenges (HCP and vitamin A deficiency) is contributing to the overall toxicity, particularly to the effect on CSFP.

The results of the comparison of vitamin A deficiency and HCP as elevators of CSFP (Table 4) provide a partial answer to this question. After 1 month on a vitamin A-deficient diet, rats had a CSFP twice as high as that of controls on normal diets (88 mm saline *v.* 40 mm), whereas rats given HCP (50 mg/kg) for only 2–3 days had a very pronounced elevation (251 mm saline). If HCP is an antivitamin A substance and its mode of action involves depletion, it is unlikely that there would be such great differences in the magnitude and time required for the two types

of CSFP response. Secondly, supplementation or repletion with massive doses of vitamin A, as described in earlier experiments, might be expected to be far more effective than they actually were, if in fact depletion by HCP plays a major role. Finally, although analysis of vitamin A in the brain was attempted without success, analysis of the vitamin in the liver at the peak of the HCP effect showed no diminution of vitamin A levels. This evidence suggests that the liver is not the locus for any vitamin A depletion effected by HCP, although the remote possibility still exists that this could occur in the brain in 2–3 days.

From a practical point of view, the LD_{50} study indicates that vitamin A pretreatment does not offer a useful degree of protection against single massive doses of HCP. Symptomatic treatment involving the lowering of elevated CSFP with hyperosmotic substances such as urea plus invert sugar has been shown to be effective in the temporary relief of paralysis in the cat (Hanig *et al.* 1973a,b & 1976).

In conclusion, vitamin A pretreatment offers partial protection, at certain doses and regimens, against the rate of development of toxic signs and early deaths due to daily HCP dosing, but the end result in terms of survival is often unchanged. Susceptibility to HCP is markedly increased in the presence of vitamin A deficiency, probably through an additive effect on CSFP via separate actions at different loci in the brain. Although HCP does not appear to be an antivitamin substance, the nutritional status of the animal with respect to vitamin A does appear to play an important role in the animal's susceptibility to the neurotoxic effects of HCP.

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ANTIPERSPIRANT AEROSOLS: AIRWAY RESPONSES FOLLOWING ACUTE AND SUBCHRONIC EXPOSURE

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Abstract—Objective ventilatory function tests conducted periodically over a 31-day period have shown that axillary application of an antiperspirant spray product twice daily causes no significant changes in airway dynamics in healthy young adults. The safety of these products in other individuals, particularly those with hypersensitivity disease of the airways remains to be demonstrated.

INTRODUCTION

The inhalation toxicity of aerosol consumer products has become a matter of growing medical concern. One recent study (Zuskin & Bouhuys, 1974) demonstrated that acute exposure to hair sprays caused transient, reversible narrowing of the small airways. On the basis of this finding, it was suggested that hair sprays may play a causal role in some cases of asthma and chronic bronchitis. Although the potential for incidental inhalation exposure also exists during application of antiperspirant aerosols, the effects of these products on ventilatory function have not been studied. Therefore we undertook an investigation of both the acute and subchronic airway responses to an antiperspirant aerosol in a group of healthy young adults.

EXPERIMENTAL

Test subjects. Twelve 22–31-yr-old subjects, nine women and three men, participated in the study. The purposes of the project were explained in detail to all subjects, and their informed consent was obtained on this basis. Two of the women and one of the men smoked cigarettes regularly; the others were non-smokers. Before and at the conclusion of the study each subject received a thorough physical examination, which included a blood and urine analysis, a 12-lead electrocardiogram and a chest X-ray. The finding of one or more clinical abnormalities during the initial examination was considered sufficient ground for excluding an individual from the study. All of the subjects lived in the same metropolitan area and all denied respiratory symptoms, a history of lung disease or any unusual exposure to air pollution.

Design of study. Prior to participation in the study, each subject was trained in the proper performance of a forced expiratory manouvre and instructed to discontinue all use of cosmetic aerosols 48 hr before reporting to the laboratory for day 0 of the study. On day 0, pulmonary function tests were conducted in triplicate and the average value of each index (see later) was recorded. Each subject was then given a 6-oz pressurized can of an unscented antiperspirant spray containing 5% aluminium chlorhydrate in a 40:60 mixture of dichlorodifluoromethane (Freon 12)–dichlorotetrafluoroethane (Freon 114). The subjects were instructed to remove their upper garments and to apply the product twice daily at 4-hr intervals for 30 days by directing the spray towards each axilla for 3 sec while breathing normally. On days 1–5 and on day 30, pulmonary-function tests were performed before and 5, 15, 60 and 120 min after the first daily exposure, for assessing the acute effects of exposure. Use of the spray was stopped on day 30 and all subjects reported back to the laboratory on day 31 for a final series of pulmonary-function tests. Subchronic effects were evaluated by comparing the mean pulmonary function values recorded on day 0 with the mean values recorded at 0 hr on days 2, 3, 4, 5, 30 and 31. The results were analysed statistically using a one-tail t test for paired data.

Evaluation of airway responses. Maximum expiratory flow was measured as a function of lung volume. Subjects inspired maximally to total lung capacity and then expired forcefully to residual volume. Flow was measured at the mouth with a Fleisch no. 4 pneumotachograph used in conjunction with a Validyne MP45 differential pressure transducer. A volume record was obtained by integrating the flow signal electronically. In order to standardize lung-volume history, each recorded effort was immediately preceded by a maximal inspiration and a return to the resting expiratory level. From time-based photographic records of the flow and volume signals, several indices of pulmonary function were computed. These were forced vital capacity (FVC), forced expiratory volume in 1 sec (FEV_1), maximum expiratory flow rate (MEFR), maximum expiratory flow rate at 50% of vital capacity ($MEF_{50\%}$) and maximum expiratory flow rate at 25% of vital capacity ($MEF_{25\%}$). Because the last two measurements were known to be influenced by changes in total lung capacity, preliminary tests were conducted to determine whether acute exposure to an antiperspirant aerosol would lead to changes in total lung capacity. Through the use of a constant-volume whole-body plethysmograph, it was confirmed in three subjects that functional residual capacity and inspiratory capacity (the two components of total lung capacity) remained unchanged for up to 2 hr following a 6-sec exposure.

Control values. Since no specific component of the antiperspirant preparation was under suspicion, it was not considered appropriate to include a parallel control group in the experimental design. Instead, the protocol called for the use of the product to be discontinued in the event of an adverse clinical response, for the subject to be followed until pre-exposure values had returned and then for aerosol administration to be reinstituted to confirm this as the primary precipitating factor in the initial reaction.

RESULTS

Acute effects of exposure to the antiperspirant spray were assessed on days 1–5 and again on day 30 by comparing mean pulmonary function values at 0 hr with those obtained 5, 15, 60 and 120 min after exposure.

Figure 1 shows the experimental results obtained on day 3. Similar results were obtained on the other 5 days on which acute responses were evaluated. No statistically significant changes occurred in any of the measured indices of pulmonary function during the 2-hr interval following exposure, and none of the subjects experienced wheezing, chest tightness, or other subjective symptoms of respiratory distress. The results of the more prolonged investigation are presented in Fig. 2. Recorded changes in pulmonary function over the 31-day period were small and statistically insignificant. On day 30, three subjects showed a moderate decline in $MEF_{25\%}$, but in each case subsequent testing later in the day yielded values very close to those recorded on day 0. The results of the clinical tests (blood and urine analysis, electrocardiogram and chest X-ray) performed on the final day of the study also failed to reveal any changes that could be attributed to the use of the antiperspirant product.

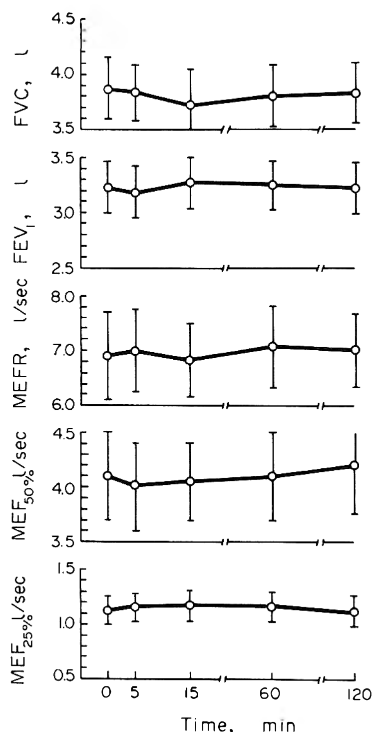

Fig. 1. Changes in ventilatory function following a 6-sec exposure to antiperspirant aerosol on day 3 of the study. Values are means ± SEM (bars) for a group of 12 subjects.

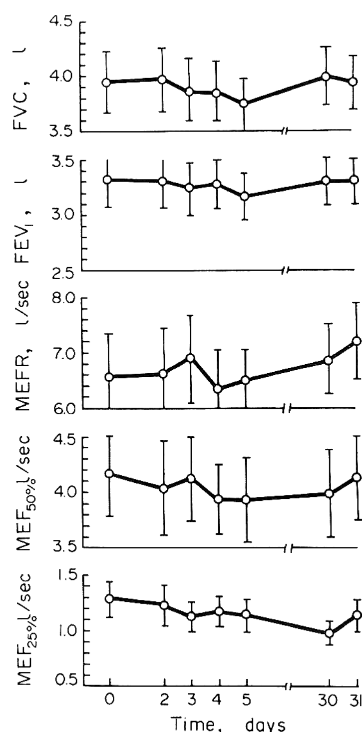

Fig. 2. Changes in ventilatory function during 1 month of twice-daily exposure to antiperspirant aerosol. Values are means ± SEM (bars) for a group of 12 subjects.

DISCUSSION

When assessing airway responses to environmental inhalants, it is important to select tests that are sensitive enough to detect changes in small-airway resistance. Since the small airways normally contribute only about 10% to the total airways resistance (Macklem & Mead, 1967), obstruction in peripheral regions of the tracheo-bronchial tree may have little influence on conventional spirometric parameters. In analysing the flow-volume data, we took into consideration the fact that as lung volume diminishes the resistance offered by small airways in proportion to that due to large airways increases. Therefore, we reasoned that measurements of $MEF_{25\%}$ and $MEF_{50\%}$ could be used as expressions of small-airway calibre, while measurements of MEFR and FEV_1 could serve as indicators of large-airway calibre. On this basis we concluded that exposure to the antiperspirant spray had no substantive effects on either the central or peripheral airways. Further, since maximum expiratory flow rates at lung volumes below about 70% of the total lung capacity are determined by the static recoil pressure of the lungs and the resistance of non-compressed intrathoracic airways (Mead, Turner, Macklem & Little, 1967), our data provide indirect evidence that the elastic properties of the lungs were not altered by exposure to the antiperspirant spray.

The failure to observe significant changes in airway dynamics following exposure to antiperspirant aerosol is in contrast to the marked and consistent reduction in expiratory flow that occurs immediately after exposure to hair spray (Zuskin & Bouhuys, 1974). This difference in airway responsiveness to the two products suggests that something other than the fluorocarbon propellants is responsible for the broncho-constricting effect of hair sprays. Alternatively, it could be argued that the duration of acute exposure to these cosmetic aerosols is an important determinant of their bronchomotor activity. The data currently available are insufficient to establish whether or not such dose–response relationships exist. In the study dealing with hair sprays, the duration of exposure was 20 sec, as opposed to 6 sec in the present antiperspirant study. Nonetheless, the results of the two investigations are comparable to the extent that the exposure times were approximately those encountered by consumers in the day-to-day usage of the two products.

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PRELIMINARY NEUROLOGICAL EVALUATION OF GENERALIZED WEAKNESS IN ZINC PYRITHIONE-TREATED RATS

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Abstract—Male rats were fed *ad lib.* a diet containing 250 ppm zinc pyrithione for 10 days and were then given the basic, untreated, diet for the rest of the study. Initial clinical signs of locomotor abnormalities were observed after the rats had been on the treated diet for 8–10 days. Progressive hind-limb weakness with accompanying muscle atrophy was a constant finding. Muscles of the forelegs and the chest appeared normal and showed almost normal function. The back muscles seemed involved, since kyphosis developed without skeletal abnormality. Weight loss was significantly greater and recovery significantly slower in treated rats than in matched pair-fed controls. Neurophysiological studies of sciatic-nerve conduction velocities failed to show differences between treated and control animals. A significant difference in contraction was noted between normal and affected muscles in that treated animals with marked muscle atrophy had a weaker contractile response to a greater electrical sciatic-nerve stimulus than had the controls. This study suggests that the lesion is at the subcellular level and the muscle atrophy may be caused by a basic abnormality in neural conduction, by dysfunction at the myoneural junction or in the muscle-fibre membrane or by a change at the molecular level in the muscle fibres.

INTRODUCTION

The water-insoluble zinc salt of 1-hydroxy-2(1*H*)-pyridinethione—zinc bis-(1-hydroxy-2(1*H*)-pyridinethionate), commonly known as zinc pyrithione (ZnPT)—is a broad-spectrum antimicrobial and antifungal agent‡ used as an antiseborrhoeic ingredient in hairdressing preparations and shampoos. The toxicity of ZnPT given by different routes of administration has been studied extensively in several species of animal and the toxic effects have been described (P. S. Larson, personal communication 1958; Adams, Wedig, Jordan, Smith, Henderson & Borzelleca, 1975; Collum & Winek, 1967; Delahunt, Stebbins, Anderson & Bailey, 1962; Moe, Kirpan & Linegar, 1960; Nolen, Patrick & Dierckman, 1975; Okamoto, Hasegawa & Urakubo, 1967; Opdyke, Burnett & Brauer, 1967; Snyder, Buehler & Winek, 1965; Wedig, Goldhamer & Henderson, 1974; Wedig, Kennedy, Jenkins, Henderson & Keplinger, 1975; Winek & Buehler, 1966).

The pyrithiones have been shown to have a high inherent toxicity when given orally on a long-term basis to non-primates (P. S. Larson, personal communication 1958; Snyder *et al.* 1965). For example, long-term feeding studies with zinc pyrithione in rats clearly established a condition of generalized skeletal muscle wasting and weakness, particularly of the hind quarters, and this could be so severe that the affected animal was incapable of standing or walking. However, Snyder *et al.* (1965) did not observe any of these toxic signs when ZnPT in a shampoo base was intubated into rats and rabbits or when it was applied dermally in a shampoo base to rabbits.

The study reported here was undertaken to investigate possible systemic effects of ZnPT and to investigate in treated rats any impairment in the neural transmission in the sciatic nerve and its branches that might contribute to the observed skeletal muscle paresis.

EXPERIMENTAL

Animals and treatment. Male rats of the CD strain (Charles River Breeding Laboratories, Wilmington, Mass.), weighing 250–300 g, were divided into 33 pairs matched by weight and were housed in individual plastics boxes containing cellulose bedding. Water was supplied *ad lib.* from individual water bottles. ZnPT was given in a homogeneous mixture of ground rat chow at a concentration of 250 ppm. This diet was based on the results of prior studies (D. D. Dearwester & G. R. Johnson, personal communication 1974), which determined the dose–latency relationship between dietary concentrations of ZnPT and the appearance of profound paresis within 10–15 days.

All rats were acclimatized to the laboratory environment for 1 wk, during which time daily records

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‡See Olin Technical Bulletin AD 1372–667; *Zinc and Sodium Compounds of Omadine®*.

of weight and food consumption were kept. During the 10-day treatment period, pair-fed controls were given an amount of untreated ground food which was equal by weight to that eaten by the treated partner during the previous 24 hr, and pair-feeding was continued during the subsequent 53 days when the treated animals were fed the untreated rat chow. During the course of the experiment, the water bottles of affected animals were laid inside the cage next to the food jar to permit each prostrate animal access to food and water.

Pathology. Three rats treated with the diet containing 250 ppm ZnPT and their pair-fed controls were killed, exsanguinated from the abdominal aorta, autopsied and examined for gross and microscopic changes. Tissues examined microscopically were the adrenals, pancreas, thyroid, parathyroid, pituitary, bone marrow, spleen, heart, kidneys, bladder, liver, lung, skeletal muscle, eye, brain (four levels), spinal cord (cervical, thoracic and lumbar), the sacral and brachial plexus and the femoral, sciatic, radial, medial and ulnar nerves.

Clinical chemistry. Blood specimens were collected from all treated and pair-fed controls for determination of white blood cells, with a differential count (3), glucose (30), blood urea nitrogen (3), potassium (29), sodium (20), chloride (26), serum glutamic–oxalacetic and glutamic–pyruvic transaminases (3), creatine phosphokinase (30), cholinesterase (6), pH (8), and partial pressures of O_2 and CO_2 (8), the total number of analyses in each case being indicated in parenthesis.

Myotoxicity. The gross myotoxic effects of ZnPT were investigated during and after the onset of generalized weakness in 12 pairs of treated and pair-fed control rats. At autopsy, the hind limbs of each animal were skinned and amputated at the coxa-femoral joint. The overlying muscles and fascia were excised or removed to expose the soleus muscle, and the entire leg was immersed in 10% formalin. After fixation for 24 hr, the fixed soleus muscle was dissected free by severing its fibrous attachments to the Achilles tendon and the fibulae. The freed muscle was blotted dry and weighed to the nearest 0·1 mg. It was placed in formalin and later prepared for longitudinal and transverse sectioning. A specimen of gastrocnemius muscle was taken for microscopic examination at the same time.

Neurophysiology. General anaesthesia was induced and maintained by dripping Metofane® onto an inhalation nose cone. The sciatic nerve and the muscles of the hind limb were exposed by a lateral skin incision from the greater trochanter to the tibiotarsal joint. The skin was separated from the underlying tissue by blunt dissection and the lateral rectus muscle was reflected back, exposing the sciatic nerve, which was carefully stripped from its supporting tissue. Once the nerve was freed, a suture was passed under the proximal end and tied, and the nerve was cut just distal to the knot as close to the spinal cord as possible. Additional sutures were used to lift and suspend the edges of cut skin vertically to form a pool which was filled with mineral oil warmed to body temperature to prevent the exposed nerve and muscles from drying.

Two pairs of platinum–iridium wire electrodes were positioned to permit the sciatic nerve to lie in the hooks formed at the end of the electrodes without distorting the natural course of the nerve. The pairs of electrodes were kept as far apart as was possible along the nerve while preventing contact of the nerve with surrounding tissue to avoid current spread. The pairs of electrodes were connected to a monophasic square wave stimulator so that the cathode was nearest the muscle. Inter-stimulus distances were measured between the cathode electrodes. The electrodes in each pair were less than 1 mm apart.

Stimulus parameters of 1 pps, 1 msec pulse width between 0·5 and 1·0 mA were used to elicit the muscular response. When the location and effectiveness of the proximal and distal stimulating electrode pairs had been verified, the recording system was connected. Approximately 1 mm of the inner wire was bared and extended beyond the bared end of the outside cannula, providing a concentric, bipolar electrode which was used to record from several sites in the same or adjoining muscles simply by withdrawing and repositioning the electrode by means of the micromanipulator in which it was mounted. The muscle potentials were first amplified and isolated by a Grass Model P15B A.C. preamplifier, then displayed on a Tektronix Model D11 dual-beam storage-oscilloscope, and, finally, recorded on Polaroid Type 107 film with a Tektronix C-5 Oscilloscope Camera. The onset of stimulation was used to trigger the cathode ray tube (CRT) sweep automatically. By convention, the onset of muscle response was defined as that point at which the CRT curve first deviated consistently from the baseline for six or more superimposed sweeps.

During the course of eliciting and recording muscle potentials with electrical stimulation of the sciatic nerve, the stimulus intensity was kept at or slightly below the level required for a maximum response. Similarly, the oscilloscope amplification was adjusted to yield nearly equal-sized muscle potentials (i.e. nearly equal deflection from the baseline).

After completion of several conduction velocity measurements, one stimulating electrode pair and the recording electrode were removed. The gastrocnemius muscle was retracted to expose the soleus muscle beneath it and, at the distal insertion of the single head of the soleus muscle into the Achilles tendon, a small hook was sutured onto the muscle surface and the muscle was cut free of the tendon. The muscle was then attached by means of the hook to a piezoelectric strain gauge mounted in a universal manipulator, the output of which was amplified through a bridge circuit for CRT display. Once the strain-gauge position and muscle tension were finely adjusted, the intensity of neural stimulation was set at the level that yielded the maximum contraction without overdriving or tetanizing the muscle. After that the CRT amplification was adjusted to yield a maximum single pulse deflection from baseline. The measure, then, of soleus muscle contractability or force pulling against the strain gauge was the intensity of stimulation required to elicit a maximum contraction and the level of amplification needed to produce similar traces (voltage deflections) comparing treated animals with pair-fed controls. Calibration of the CRT deflection with a known set of weights attached to the piezoelec-

tric strain gauge permitted a transformation from mV/cm (CRT gain scale) directly to force (g) of muscle tension (see below).

RESULTS

General responses

The rats given the treated diet showed no changes in gait or behaviour until after an average of 8 days (range 6–13 days). At this point, they began to show the first signs of an impending weakness which appeared as a splayfooted stiff gait and slightly abducted rear limbs. With continued treatment, this condition grew worse until, usually within a further 48 hr, the animal became incapable of standing, developed a mild kyphosis and was able to move only by dragging itself along the bottom of the cage by its fore limbs. For the purposes of this report, the *onset* of generalized weakness is designated as the first day on which the animal was no longer able to stand. Beyond this point, the animals in this study died or were considered moribund and were killed for further laboratory examination after an average of 5·9 days (range 3–12 days). This post-weakness survival period could be extended by adopting husbandry practices, such as by making food and water more accessible.

The affected animals continued to be alert and responsive to auditory and visual stimuli. Breathing patterns were normal and electrocardiograms taken from three severely affected animals indicated that cardiac conduction was unchanged. Faeces were normal in colour and consistency. Badly affected animals had urine-soaked fur in their inguinal area. Since no indication of incontinence was detected, it was assumed that the urine soaking was due to the animal's immobility. All severely affected animals showed penile prolapse, which was never seen in control animals. Except for ruffled fur and inactivity, control animals showed no response to their reduced food intake.

Weight loss began to occur within 24 hr of the onset of treatment. Both treated and pair-fed controls showed weight losses, which amounted to 26% of the pretreatment weight in the treated animals and 22% in the control group. The most striking weight loss occurred between the start of treatment and the onset of the previously described signs. During this period, the treated rats incurred an average 11% weight loss, compared with 5% in control rats. A further weight loss, which occurred following the onset of clinical signs, averaged 17% in treated animals and 14% in control rats. During the latter period, food consumption fell even further, presumably because of the difficulty a severely affected animal encountered in reaching food jars. Consequently, consumption of ZnPT also decreased. The average consumption of ZnPT throughout the treatment period was 9·9 mg/kg/day (range 3·9–23·8 mg/kg/day). Before the onset of clinical signs, consumption averaged 10·9 (3·5–14·6) mg/kg/day, while from the onset to the end of the study this average was 5·3 (0·7–25) mg/kg/day.

Eight treated rats and their pair-fed controls were used to investigate the reversibility of toxicity. On day 10, by which time marked clinical signs were present in all treated rats, ZnPT was withdrawn and both groups were placed on unadulterated food *ad lib*. Weight loss in the treated animals continued for 3–4 days following withdrawal of the treated diet, whereas the pair-fed rats immediately began regaining their lost weight. More striking was the slower recovery of the mean body weight of the treated animals during the 35 days after treatment (Fig. 1), the weights remaining significantly below those of the control rats.

Pathological changes

The only consistent drug-related change was a direct reduction in the diameter of muscle fibres from the soleus, gastrocnemius and sacrospinalis. This atrophy appeared to be due primarily to a loss of cytoplasm. The quantitative reduction in skeletal muscle mass was reflected in the weight of fixed soleus muscles from seven pairs of treated and control rats. The mean weight of the soleus muscles (formalin-fixed) from ZnPT-treated rats that had shown locomotor dysfunction for 4–11 days was significantly lower than that of pair-fed control rats (85·9 *v.*

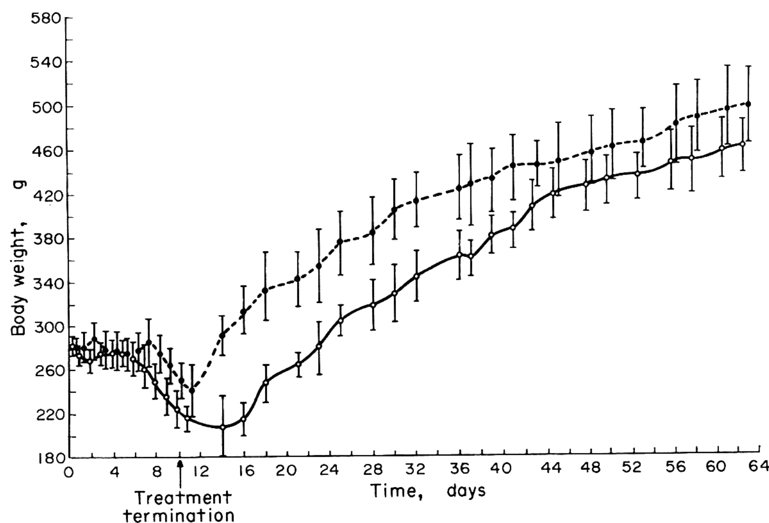

Fig. 1. Mean body weights (±1 SD) of groups of 16 treated (o) and pair-fed control (•) rats recorded during a 10-day period of ZnPT feeding and for 53 subsequent days when both groups were given the basal diet.

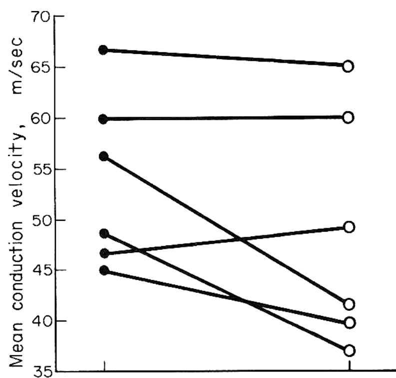

Fig. 2. Mean conduction velocity in the sciatic nerve of each treated animal (●) linked to that of its pair-fed control (○).

138·7 mg, respectively). However, when a separate group of five pairs of treated and central rats was examined at the onset of paresis (i.e. at the first signs of hind-limb dragging), there was no difference between the soleus muscle weights of treated rats and pair-fed controls.

Histopathological examinations of other organs and laboratory evaluations of blood specimens all failed to demonstrate any differences between ZnPT-treated animals and their pair-fed controls.

Neuromuscular conduction studies

There is sufficient overlap in the sciatic nerve conduction velocities from treated and control rats (Fig. 2) to eliminate the possibility that a neural impairment severe enough to include the larger-diameter fibres in the sciatic nerve could be responsible for the generalized weakness. Normal function in the small-diameter fibres remains to be examined. Repetitive conduction-velocity traces from treated and control rats were consistently reproducible (Fig. 3).

The velocity of conduction in the sciatic nerves (Fig. 2) did not show any change as a result of ZnPT treatment. However, the stimulation intensity required to maintain maximum contraction, corrected for similar sized muscle potentials, demonstrated that while the conduction velocities did not appear to be affected, the treated animals required 5–7 times the level of neural stimulation to produce generally weaker muscular contractions.

A similar analysis of the contractile force of the soleus muscle against a calibrated piezoelectric strain gauge showed that maximum tension was achieved between a narrow stimulus band of 0·2–0·3 mA in both treated and control rats. Yet the contractile force was 2–20 times lower in the treated rats. Conversion of CRT deflection size (amplitude of response) and gain scale (mV/cm) to force (g) was achieved by calibrating the piezoelectric transducer with known weights. In these experiments, 1 mV amplitude was found to equal 0·33 g, or 1 g force equalled 3 mV deflection. Thus, response amplitude (cm) × gain scale (mV/cm) × 0·33 (g/mV) = force (g).

In three sets of strain-gauge experiments, there was no overlap between the muscle responses of treated and control rats in terms of the force (mg) of contraction of the soleus muscle (Fig. 4). The pair-fed control rats showed significantly greater soleus muscle strength than the ZnPT-treated rats.

DISCUSSION

Repeated ingestion of ZnPT by rats leads to a generalized weakness which can be so severe that the affected animal is incapable of standing or walking. The studies described in this report investigated functional changes in two general areas that might provide an explanation for this weakened condition. The first part of the study was concerned with systemic effects and the second with neural transmission in the

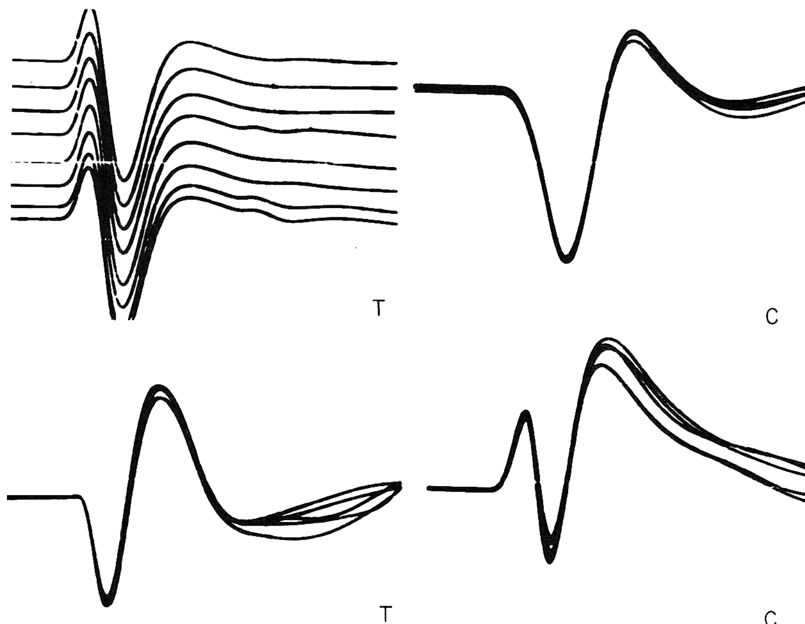

Fig. 3. Four samples of characteristic muscle potentials elicited by sciatic-nerve stimulation, in treated (T) and pair-fed control (C) animals, showing the reliability and superimposability of the multiple traces.

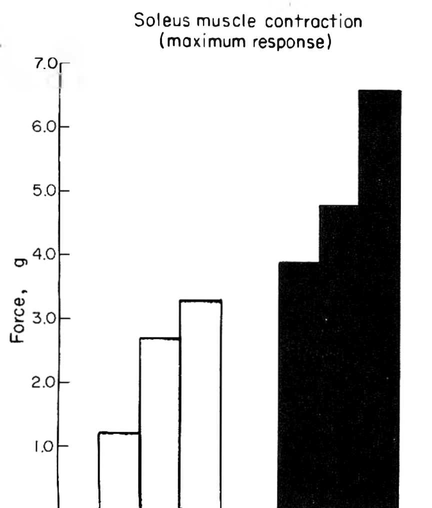

Fig. 4. Force of maximal soleus-muscle contraction in three pairs of treated rats (□) and pair-fed controls (■).

sciatic nerve and its branches, and measurements of the strength of contraction of the isolated soleus muscle.

In designing the study, a number of chemically-induced systemic deficits were postulated and laboratory analyses were selected that would contribute to the support or rejection of these hypotheses. The amount of blood collectable from each rat limited the number of determinations that could be performed and test procedures were therefore selected on the basis of such factors as the available information, data variability and correlation with clinical and pathological signs. Potential aetiologies that were considered and discounted because of a lack of differences between treated and control animals were anaemia, hypoxia, acidosis, alkalosis, electrolyte imbalance, hypoglycaemia and cholinesterase inhibition.

The absence of any change in conduction velocities (Fig. 2) indicates that the sciatic nerves in the treated rats were functionally indistinguishable from those of the pair-fed controls by the methods used. This should not be taken to imply that neither the peripheral nor central nervous system is involved in ZnPT-induced paresis. The functional integrity of the myoneural junction, pyramidal and extra-pyramidal spinal-cord tracts and upper motor neuron pathways of the central nervous system in treated rats remains to be examined.

Weaker muscle contraction (Fig. 4) indicates a pronounced decrease in the muscular responsivity of the affected animals. It should be noted, however, that the size and threshold of muscle potentials are sensitive to and can be grossly affected by the placement of recording electrodes. Since nearly all the muscle responses (both electrical potentials and strain-gauge forces) were markedly lower in the treated rats, a more likely explanation for the neurophysiological findings in this study is muscle-fibre atrophy. Muscular atrophy would be expected to produce a reduction in external field (i.e. potential shift). Since a parallel body-weight loss occurred in treated and control animals until well after severe paresis had appeared, it is possible that ZnPT caused a chemical lesion that resulted in muscle atrophy beyond any produced in the controls by weight loss alone.

Since these preliminary studies showed that muscle size was nearly normal in affected animals up to the first day of clinical illness, neurophysiological responses need to be assessed at or before onset, during the prodromal state of the illnesss, to locate the primary site of action.

In order to discover the primary site and mechanism of action, a stepwise series of experiments at each level of the affected musculo-skeletal system is indicated. If the dysfunction proves to be neurogenic, a study of the axoplasmic flow pattern and biochemical assays of whole nerve or teased fibres may afford an insight into ZnPT's axonal effects. If the disorder is junctional, anatomical studies of the neuromuscular junction should be pursued. The need for studies of end-plate potentials would be indicated by a neurophysiological defect, and, if the lesion is muscular, biochemical analysis of the contractile proteins, calcium uptake and release by the sarcoplasmic reticulum, and electron-microscopic freeze-fracture of the muscle membrane may lead more directly to the underlying mechanism.

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ABSORPTION, EXCRETION AND TISSUE DISTRIBUTION OF 2-PYRIDINETHIOL-1-OXIDE

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Abstract—Sodium pyrithione and zinc pyrithione, labelled with ^{35}S, were administered to rats, rabbits and monkeys, either orally or ip. Rats with cannulated bile or thoracic ducts were used in some experiments. Excreta and carcasses were analysed for radioactivity to determine the metabolic fate of the pyrithione moiety. Both sodium and zinc pyrithione were absorbed into the circulation to the extent of 80–90%. The ^{35}S was excreted rapidly in the urine (20–50% in 6 hr, 70–85% in 48 hr), but a moderate amount (*c.* 15%) was excreted in the bile. Small amounts (2–9%) of the ^{35}S remained widely distributed in the body 72 hr after administration.

INTRODUCTION

Salts of 2-pyridinethiol-1-oxide are useful as antifungal agents, and the zinc salt, commonly known as zinc pyrithione (ZPT), is used as the active component of some anti-dandruff shampoos. Both ZPT and the corresponding sodium pyrithione (NaPT) have received extensive toxicological investigations (Brauer, Opdyke & Burnett, 1966; Collom & Winek, 1967; Moe, Kirpan & Linegar, 1960; Opdyke, Burnett & Brauer, 1967; Snyder, Buehler & Winek, 1965; Winek & Buehler, 1966). Previously reported toxic manifestations include ocular effects in dogs dosed with 25 mg ZPT/kg/day for 14 days and skeletal muscle paralysis in rats receiving 20 mg ZPT/kg/day in their feed for 7 days. Rhesus monkeys, after six daily doses of 10 mg ZPT/kg, showed only emesis and diarrhoea, and a monkey that received 10 mg ZPT/kg on 5 days/wk for 16 wk showed no adverse effects.

Since the normal use of products containing ZPT brings the compound into contact with the skin, there have been several studies of the absorption, distribution and excretion of topically applied ZPT and NaPT (Howes & Black, 1975; Howlett & van Abbe, 1975; Klaassen, 1976; Parekh, Min & Golberg, 1970). These studies have shown that intact skin is penetrated by ZPT to a very slight degree, and by NaPT to a somewhat greater degree. The organic and inorganic portions of the ZPT molecule are metabolized separately; the zinc evidently enters the systemic zinc pool, while the pyrithione is rapidly excreted in the urine, with small quantities appearing in the bile. Min, Parekh, Golberg & McChesney (1970) and Howlett & van Abbe (1975) reported that the pyrithione is excreted largely as pyridine-*N*-oxide-2-sulphonate.

NAPT and ZPT are disposed of in much the same way when administered iv or ip as when they are administered topically, but much higher systemic exposures are possible by these routes since the chemicals do not have to penetrate the skin.

Klaassen (1976) gave oral doses of [^{14}C]ZPT and [^{65}Zn]ZPT to rabbits, and observed appreciable absorption. The pattern of disposition was parallel to that observed with ip exposure, since ^{14}C, but not ^{65}Zn, was excreted in the urine soon after dosing. Kabacoff, Fairchild & Burnett (1971) reported that rabbits excreted the pyrithione as its glucuronide after oral dosing with ZPT.

Although most attention has been directed toward the metabolism of topically applied pyrithiones, in view of their intended use, it is possible that misuse of products may lead to oral exposure. We were prompted, therefore, to investigate the metabolism of orally ingested ZPT and NaPT.

EXPERIMENTAL

Materials. NaPT and ZPT, 97·4 and 97·2% pure, respectively, were obtained from Olin Corp. (Stanford, Conn.). [^{35}S]ZPT (8 μCi/mg) was prepared in our laboratories by interacting 2-chloropyridine-*N*-oxide with $NaH^{35}S$. Thin-layer chromatography on Silica Gel G (E. Merck AG, Darmstadt, Germany) plates developed with chloroform–2-butanone–acetic acid (72:20:8, by vol.) indicated its purity to be 97%.

For oral administration, [^{35}S]ZPT was prepared in the syringe used for gavage by mixing an aqueous solution of [^{35}S]NaPT with an aqueous solution containing a 5% molar excess of zinc acetate. Dosing with the [^{35}S]ZPT was followed by dosing with the small amount of water used to rinse the syringe. Doses for ip administration were 1–3 ml; oral doses were about 3 ml for the rat and 5 ml for the rabbit and monkey.

Animals. Male Sprague–Dawley rats, male New Zealand rabbits and male rhesus monkeys were used. Immediately after dosing, the animals were placed in stainless-steel metabolism cages for the separate collection of faeces and urine. Cannulated rats were housed in restraining cages during collection of body fluids. Animals were anaesthetized with sodium pentobarbitone (5 mg/kg, ip) for surgical procedures and for terminal blood sampling by cardiac puncture.

Radioassay. Excreta and tissue samples were assayed for ^{35}S by liquid scintillation spectrometry. Urine, bile and lymph were counted after direct addition of 15–20 ml scintillator fluid (4 g PPO and 0·05 g DM-POPOP/litre toluene) containing Cab-O-Sil

Table 1. *Metabolism experiments with sodium* [^{35}S]*pyrithione*

	Data for experiment no.									
	1	2	3	4	5	6	7	8	9	10
Test animals										
Species	Rat	Rat	Rat	Rat	Rat	Rat	Rat	Rat	Rabbit	Monkey
No.	3	3	3	3	3	4	3	1	3	1
Dietary status*	normal	fasted	fasted	fasted	normal	prefed	normal	normal	fasted	fasted
Mean body weight (g)	179	238	100	297	252	120	194	258	2267	5085
Dosage										
Route†	oral	oral	oral	oral	oral	oral	ip	ip	oral	oral
Amount (mg)	5	5	2·5	7·5	48	5	5	33·6	25	50
Distribution of ^{35}S (% of dose)										
Urine: 0–6 hr	28	45	50	45	20	58	70	54		80
6–12 hr	18	9·3	11	13	8·6		7·1	12		
12–24 hr	15	6·5	8·2	8·8	25	18‡	3·9	12	78§	8·9‡
24–48 hr	14	10	10	10	21	7·8	3·1	8·6	6·4	2·5
48–72 hr	4·8	3·7	3·2	4·3	11	2·4	1·8	3·9	2·9	—
Total....	80	75	83	82	86	86	86	90	87	90
Faeces: 0–24 hr	6·2	8·0	3·2	2·0	1·0	2·7	4·8	1·0	4·2	1·0
24–48 hr	12	5·4	2·5	3·0	4·9	2·5	1·3	1·2	3·0	0·2
48–72 hr	1·1	1·1	0·5	0·8	3·4	1·2	0·8	<0·4	0·7	—
Total....	19	14	6·2	5·8	9·3	6·4	6·9	2·2	7·9	1·2
Cage washings	0·3	0·2	—	—	—	—	0·2	—	—	—
Gut washings	0·4	0·5	0·3	0·4	1·3	0·2	0·2	0·3	1·3	—
Gut	0·2	0·2	0·2	0·2	0·4	0·1	0·1	0·2	0·3	—
Liver	—	—	0·2	0·3	0·6	0·1	—	0·4	0·2	0·1
Kidneys	—	—	0·04	0·09	0·38	0·03	—	0·20	0·06	0·02
Carcass	4·0	4·5	2·8	4·1	9·2	2·1	2·2	3·7	2·3	—
Tissue level of ^{35}S (μg NaPT/g wet tissue)										
Blood	—	—	0·5	1·4	23	0·8	0·4	<1·0	0·4	0·4
Carcass	1·3	1·0	0·8	1·6	24	1·3	0·7	6·4	0·4	—
Gut	0·8	—	0·4	1·1	—	—	—	—	—	—
Kidney	—	—	0·7	2·6	82	1·2	—	28	0·8	0·72
Liver	—	—	0·8	1·8	24	1·2	—	6·0	0·7	0·6
^{35}S accounted for (% of dose)	104	95	92	93	107	95	96	97	100	93‖

*In experiments 2, 3, 4, 9, and 10, the animals were fasted for 18–24 hr before dosing. In experiment 6, the animals were preconditioned by feeding a ration containing 235 ppm NaPT for 15 days before dosing.

†Oral doses were administered by stomach tube.

‡Combined 6–24 hr value.

§Combined 0–24 hr value.

‖Carcass values were not determined.

(Cabot Corp., Boston, Mass.; 34 g/litre) to a sample aliquot of ⩽1 ml. Heparinized blood samples were bleached with benzoyl peroxide, treated with NCS (quaternary ammonium hydroxide (0·6 N) in toluene supplied by Nuclear Chicago Corp.), and counted in scintillator fluid. Faeces, gastro-intestinal tract, and gastro-intestinal contents were minced or homogenized, and representative samples (100–250 mg wet weight) were treated overnight with 2 ml NCS solution at 100°C, after which 15 ml scintillator solution (8 g PPO and 0·5 g DM-POPOP/litre toluene) was added for counting. Representative slices of other soft tissues and a portion of homogeneous material from the ground frozen carcasses were analysed by the NCS method.

Experimental procedures. The excretion and distribution of [^{35}S]NaPT were studied in ten experiments, which are summarized in Table 1. [^{35}S]NaPT was administered orally or ip to rats and orally to rabbits and monkey. The excreta passed by the animals were assayed for ^{35}S to determine the route and rate of excretion, and the animals were killed and various tissues were analysed to measure the distribution of ^{35}S. Some animals were fasted overnight before dosing, some had normal stomach contents when dosed, and some were preconditioned by feeding them a ration containing non-radioactive NaPT for 15 days before dosing. Both the size of the animal and the dose were varied.

A second series of experiments (11–15) was designed to investigate in detail the tissue distribution of ^{35}S after administration of [^{35}S]NaPT to rats. These experiments, summarized in Table 2, resembled the first series in general procedure except that more and different tissues were analysed. In a third series of experiments, surgically altered rats were used to study the routes of absorption and excretion of ^{35}S from [^{35}S]pyrithione. Three of these experiments (16–18) are described in Table 3.

In experiment 16 a plastic cannula had been inserted into the bile duct of each animal, and back flow from the intestine was prevented by ligation of the bile duct distal to the insertion point of the cannula. The plastic tube was drawn through the abdominal wall, the incision was closed and the animals were allowed to recover overnight. After ip administration of [^{35}S]NaPT, bile was collected at regular intervals for ^{35}S analysis.

In experiment 17, [^{35}S]NaPT was administered orally to an animal that had been cannulated as in

Table 2. *Distribution of* ^{35}S *after administration of sodium* [^{35}S]*pyrithione to rats*

	Data for experiment no.								
	11		12		13		14	15	
No. of rats	2		2		3		3	2	
Dietary status*	fasted		normal		normal		fasted	normal	
Mean body weight (g)	224		218		268		243	192	
Dosage									
Route†	oral		oral		oral		oral	ip	
Amount (mg)	5		5		5		1·67	5	
Distribution of ^{35}S‡	**%**	**μg/g**	**%**	**μg/g**	**%**	**μg/g**	**%**	**%**	**μg/g**
Faeces			6·2		3·4			0·25	
Urine + cage washings	9·8		67		63		3·5	23	
Gut washings	18·0		7·6		2·5		26	8·2	
Gut	26		1·0		1·4		20§	5·4	
Carcass	27	8·4	17	6·7	25	6·7		16	6·0
Heart	0·09	4·4	0	0	0·1	6·4		0·06	3·0
Liver	19	110	1·4	9·0	1·6	7·4		34	152
Kidneys	2	42	0·4	11·0	0·6	12		1·1	26
Pancreas	0·7	19						0·2	5·3
Testes	0·3	4·2			0·4			0·2	3·9
Spleen	0·3	20			0·08	6·6		0·04	3·0
Muscle		3·1		7·0					3·5
Eyes	0·01	2·3						0·012	2·4
Brain	0·08	2·5			0·1	5·3		0·05	1·9
Nerve		3·2							
Fat pad		3·4							4·1
Tissue rinse	2·0							0·4	
Blood		9·1		7·2		7·6			6·8
Lungs								0·02	8·3
Soft tissue				7·3					
Skin and hair				6·2					
^{35}S accounted for (% of dose)	105		101		98			89	

*In experiments 11 and 14 the animals were fasted for 18–24 hr before dosing.
†Oral doses were administered by stomach tube.
‡For some specimens the distribution of ^{35}S is expressed as % of administered dose; for tissues of undetermined total weight the concentration of ^{35}S in a representative sample of tissue is given as μg [^{35}S]NaPT/g wet tissue.
§Analyses of separate gastro-intestinal organs gave the following results (organ, % of dose and μg NaPT/g wet tissue): Stomach 7·9 and 73; small intestine, proximal half, 5·0 and 26; small intestine, distal half, 5·1 and 23; caecum, 0·5 and 6·8; large intestine, 1·3 and 17·6.

Table 3. *Metabolism experiments with sodium* [^{35}S]*pyrithione in surgically altered rats*

	Data for experiment no.			
	16	17		18
Alteration	Bile-duct cannulation	Crossed biliary cannulation		Thoracic-duct cannulation
No. of rats	2	2 pairs		2
Mean body weight (g)	201	211		260
Dosage: Route*	ip	oral		oral
Amount (mg)	5	10		5
Duration (hr)	72	72		72
Distribution of ^{35}S (% of dose)		**Donor**	**Recipient**	
Urine	62	66	8·7	68
Faeces	15	12	0·8	16
Gut washings		0·3	0·4	0·3
Gut				0·2
Carcass	5·0	5·2	2·4	6·6
Liver	0·06			
Kidney	0·01			
Bile	14·9		4·2	
Pancreatic fluid	1·5			
Lymph				11
^{35}S accounted for (% of dose)	98	83	17	102

*Oral doses were administered by stomach tube.

experiment 16, but the effluent end of the cannula from this rat was inserted into the distal portion of the bile duct of a second rat, so that the bile from the first rat flowed into the intestine of the second rat. Bile from the second rat was collected externally through another cannula which was inserted in the proximal portion of its bile duct. Tissues of both the donor and recipient animals were analysed for ^{35}S at the end of the experiment.

Experiment 18 measured the extent of absorption of [^{35}S]NaPT through the lymphatic system. The thoracic ducts of these animals were cannulated the day before [^{35}S]NaPT was administered orally. Lymph was collected at regular intervals and analysed for ^{35}S.

In another experiment, not shown in the tables, the absorption of pyrithione from the stomach was measured by surgically tying off the stomachs of three rats at the pyloric valve and then administering 1·67 mg [^{35}S]NaPT to each animal by stomach tube. After 3 hr the animals were killed, and the amount of ^{35}S remaining in the stomach was measured.

Finally, in experiments 19–21 (Table 4) the zinc salt was studied by measuring the absorption, excretion, and distribution of [^{35}S]ZPT after oral administration to fasted or non-fasted rats and to a monkey.

RESULTS

Absorption and excretion after oral administration of NaPT to rats

The results in Table 1 indicate that NaPT was absorbed extensively from the gastro-intestinal tract, and that the primary route of excretion was in the urine. Relatively small amounts of ^{35}S remained widely distributed in the body. Urinary excretion of ^{35}S was more rapid in fasted than in unfasted rats, presumably because the absorption of NaPT by unfasted animals was delayed by its interaction with gastro-intestinal contents. Although the data reveal the fate of the ^{35}S atom from [^{35}S]NaPT, they give no indication as to whether the material being measured was pyrithione or a metabolite.

Experiments, 2, 3 and 4 showed that variation of body weight between 100 and 300 g did not affect the fate of [^{35}S]NaPT in rats.

In experiment 5, the dose of [^{35}S]NaPT administered was much larger than in the earlier experiments, but the results were similar. The fraction of administered ^{35}S that remained in the carcass was slightly, but not significantly, greater than had been observed with the smaller doses. Thus the pattern of absorption and distribution appears not to be a function of dosage level.

It seemed possible that the hind-leg paralysis that has been seen in rats fed NaPT in the diet might influence the pattern of absorption and excretion. Therefore, rats were pretreated by feeding them a ration that contained 235 ppm NaPT until they developed hind-leg paralysis and showed a reduced body weight and were then dosed orally with [^{35}S]NaPT (experiment 6). The resulting pattern of excretion did not differ markedly from the pattern seen in normal fasted rats (experiment 3) with which they were comparable.

Excretion after ip dosage

In experiment 7, elimination of radioactivity in the urine was rapid and accounted for 86% of the ip dose after 72 hr. In this experiment, 7% appeared in the faeces, but, unlike the situation in the oral-dosing experiments, this could not have represented unabsorbed material. Rather, it must have resulted from contamination of the faeces with urine during collection, absorption from the peritoneal cavity through the gut wall, or introduction into the gut from bile or pancreatic fluid. In experiment 8, the much larger

Table 4. *Metabolism experiments with zinc [^{35}S]pyrithione*

	Data for experiment no.		
	19	20	21
Test animals			
Species	Rat	Rat	Monkey
No.	3	4	1
Dietary status*	fasted	prefed	fasted
Mean body weight (g)	195	138	4920
Dosage			
Route†	oral	oral	oral
Amount (mg)	5·3	5·3	53·2
Distribution of ^{35}S (% of dose)			
Urine: 0–6 hr	22	22	7·3
6–12 hr	13		
12–24 hr	12	35‡	60·0‡
24–48 hr	13	15	17
48–72 hr	6	4	4·2
Total....	66	76	89
Faeces: 0–24 hr	6·2	2·5	0·4
24–48 hr	8·6	3·0	0·2
48–72 hr	2·4	1·9	0·3
Total....	17	7·5	1·0
Cage washings	0·6		
Gut washings	0·5	0·2	0·5
Gut	0·3	0·2	0·1
Liver		0·2	0·1
Kidney		0·04	0·02
Carcass	5·3	2·1	
Tissue levels of ^{35}S as μg NaPT/g wet tissue			
Blood		1·2	
Carcass	1·6	1·3	
Kidney		1·8	0·5
Liver		1·4	0·5
^{35}S accounted for (% of dose)	90	86	91

*In experiments 19 and 21 the animals were fasted for 18–24 hr before dosing. In experiment 20, the rats were fed a ration containing 250 ppm ZnPT for 15 days before dosing.
†Oral doses were administered by stomach tube.
‡Combined 6–24 hr value.

ip dose was handled in essentially the same way as the dose given in experiment 7.

Absorption and excretion in rabbit and monkey

Because different species responded differently to NaPT, corresponding differences in patterns of absorption and excretion were sought. Rabbits and a rhesus monkey given oral doses of NaPT (experiments 9 and 10) showed a pattern similar to that of the rat. Radioactivity was almost completely absorbed from the digestive tract and was excreted rapidly into the urine with only small amounts being retained in the body after 72 hr.

Rates of absorption and excretion

Although the first ten experiments established that only a small fraction of NaPT remained in the animal's body 72 hr after administration, there remained a possibility that high concentrations might appear in specific organs. Therefore, in experiment 11 (Table 2), the animals were examined 1 hr after dosing in order to obtain additional information about the rates of absorption and excretion. The ^{35}S analyses showed that most of the radioactivity had been absorbed from the digestive tract within 1 hr, that much had already been excreted in the urine and that appreciable quantities were localized in the liver and kidney. Experiments 12 and 13 were similar except that the animals were not fasted before dosing and were kept for 18 or 24 hr before being killed. Within these periods, much larger fractions of the ^{35}S were excreted in the urine, and the quantities in the kidney and liver had decreased significantly.

Tissue distribution

In experiment 14, a closer examination was made of the digestive tracts of rats 1 hr after they had received [^{35}S]NaPT orally. Of the administered doses 20% appeared in the tissues of the gastro-intestinal tract, particularly the stomach and small intestine. It was not apparent whether the ^{35}S was in the process of absorption and thus within the tissue cells, or was bound to the tissue in a way resistant to water-washing. Experiment 15 examined the tissue distribution after ip administration of [^{35}S]NaPT. The highest concentrations of radioactivity were seen in the liver and kidney, the presumed sites of biotransformation and excretion processes. Also, substantial concentrations were seen in the digestive tract suggesting that some excretion might be occurring via the biliary pathway.

Appearance in bile and lymph

Because radioactivity was found in the gastro-intestinal tract or faeces of animals that had received [^{35}S]NaPT ip, the labelled pyrithione was administered ip to bile-duct-cannulated animals in experiment 16 (Table 3). After 72 hr, 15% of the administered dose had been excreted in the bile. In experiment 17, pairs of animals were interconnected, and bile from a donor animal, which received [^{35}S]NaPT orally, was led into the digestive tract of a recipient animal, whose bile was collected separately. After 72 hr only 83% of the administered radioactivity could be accounted for in the urine, faeces and carcass of the donor animal, but 17% appeared in the recipient animal, distributed among the urine, faeces, carcass and bile. Since only a small fraction of the radioactivity going into the recipient animal proceeded down the gastro-intestinal tract into the faeces, most of the radioactivity was absorbed from the bile that entered the gastro-intestinal tract of this animal and was recirculated back to the liver and kidney for excretion.

To study the route of absorption of NaPT from the gastro-intestinal tract, experiment 18 used two rats that had been prepared by cannulation of the thoracic duct, from which lymph was collected for 72 hr after the oral administration of [^{35}S]NaPT. Of the administered radioactivity, 68% was found in the urine, 16% in the faeces, and 11% in the lymph. Evidently, only a portion of the ^{35}S was transported from the intestinal tract into the body through the lymphatic system.

Further study of the route of absorption was made in an experiment using rats that had a ligature at the pyloric valve. (This experiment is not shown in the table). The fasted animals were given 5 mg

[^{35}S]NaPT by stomach tube. They were killed 2 hr later and the contents were washed from their stomachs. The washed stomach tissues contained 4·65% of the administered ^{35}S, and the contents of the stomach contained 43·8%. By difference, about 50% of the ^{35}S was absorbed in 2 hr from the stomachs of the fasted rats.

Comparison of NaPT and ZPT

The final series of three experiments measured the absorption, distribution, and excretion of ZPT for comparison with NaPT. These experiments are summarized in Table 4. Most of the radioactivity from [^{35}S]ZPT, like that from [^{35}S]NaPT, was absorbed from the gastro-intestinal tract, and most of the absorbed material was excreted in the urine. When the animals had been fasted before administration of ZPT, more ^{35}S appeared in the faeces than when they had been preconditioned by feeding for 15 days with a diet containing 250 ppm ZPT. The fasted rats may have absorbed ^{35}S less completely than the pretreated rats, they may have excreted more ^{35}S in the bile, or there may simply have been more contamination of the faeces by urine in this experiment.

DISCUSSION

The pyrithione moiety appears to be absorbed to the extent of 60–80% from the gastro-intestinal tract when it is administered orally as either the sodium or the zinc salt, and this moiety, or its metabolic product, is rapidly excreted in the urine by the rat, the rabbit and the monkey. The similarity of absorptions of the sodium and zinc salts suggests that the highly insoluble zinc salt may be dissociated in the stomach. Pretreatment of the animals with NaPT or ZPT does not affect the absorption or excretion. A small percentage of the pyrithione or its metabolic product is transported by the lymphatic system, with most of the material presumably being transported by the portal blood. Absorption may also take place directly from the stomach. Approximately 15% of the absorbed pyrithione is excreted in the bile.

No extended build-up of pyrithione or its metabolites was seen in any tissue, and the high concentrations of ^{35}S in the liver and kidney 1 hr after dosing are consistent with the view that pyrithione is metabolized primarily by the liver and excreted primarily through the kidney into the urine.

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PHOTOTOXICITY TESTING OF FRAGRANCE RAW MATERIALS

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Abstract—Of 160 fragrance raw materials tested for phototoxicity by a procedure involving topical application of the test agents to the skin of hairless mice and miniature swine, followed by exposure of the skin either to simulated sunlight or to near-ultraviolet light (UV-A), 21 elicited a phototoxic response. Of these, 20 were members of the botanical families Rutaceae or Umbelliferae; the single exception was from the family Verbenaceae. Several selected materials were tested, in sunlight and under three laboratory light sources, on the skin of mouse, man and miniature swine. Mouse skin was the most sensitive, reactions in human and swine skin being qualitatively and quantitatively similar.

INTRODUCTION

Photosensitized tissue responses are among the potentially adverse effects of several classes of natural and synthetic chemical compounds (Blum, 1941; Clare, 1955). Photosensitivity is an interactive process, and its occurrence depends on several variables, such as the nature of the compound, the route of administration, the light quality and quantity and the tissue condition. Photosensitization is a broad term, including such manifestations as phototoxicity (possible after one exposure to the compound and the activating light), photoallergy (involving the immune system and requiring at least two exposures) and enhanced photocarcinogenesis, which generally involves many exposures to the compound and light (Forbes, Davies & Urbach, 1976b; Forbes & Urbach, 1975).

Phototoxic responses have the appearance of exaggerated sunburn, and in terms of human morbidity they are currently the most frequently recognized of the photosensitized reactions described above. Some compounds (e.g. psoralens, salicylanilides, porphyrins) can elicit more than one of the forms of photosensitized skin reactions (Harber & Baer, 1972; Herman & Sams, 1972; Kligman & Breit, 1968; Storch, 1965), and the interrelationship of the three reactions is uncertain, partly because the field of light testing is still somewhat primitive and undergoing further development.

Phototoxicity testing is included in the safety evaluation of raw materials by the Research Institute for Fragrance Materials (Opdyke, 1973, 1974, 1975 & 1976), and several methods for identifying photosensitizing compounds have been reported (Harber & Baer, 1972; Herman & Sams, 1968; Isen & Davis, 1969; Kligman & Breit, 1968; Marzulli & Maibach, 1970). The purpose of this paper is to describe one method of screening materials for skin phototoxicity and to present a list of the materials subjected to this test. As additional materials are tested, the results will be included in subsequent monographs in this journal.

EXPERIMENTAL

Preliminary experiment

Experimental subjects. The preliminary experiment included a comparison between human and animal responses. The untanned skin on the backs of ten healthy white volunteers was treated topically with test agents, as described in the animal studies below and exposed to light. The procedure was approved by the Research Review (Human Subject) Committee of the faculty, and the subjects signed a consent form which explained the procedures and risks.

Test agents. Five fragrance materials were tested: Bergamot A (expressed 1970 crop), Bergamot B ("bergapten free"), Lime A (distilled, "psoralen free"), Lime B (expressed) and sandalwood oil (West Indies). In addition, 8-methoxypsoralen (8-MOP), dissolved in methanol, was used as a positive control. The five fragrance materials were used full strength, or as 20 or 10% (v/v) solutions in methanol and 8-MOP was used as a 0·01% (w/v) solution in methanol.

Light sources. Two of the individuals were exposed to 30 min of summer sunlight after pretreatment with the compounds; all ten were also tested with a compact-arc xenon lamp solar stimulator (Berger, 1969; Stern & Urbach, 1972) fitted with a WG320 Schott filter to exclude wavelengths of <295 nm. Substitution of a WG345 filter (transmitting >320 nm) excluded the UV-B (320–280 nm) component from the beam.

Because of the inconvenience and irregular availability of sunlight, and the tediousness of multiple small spot exposures with the compact-arc solar simulator, two other sources offering convenience and exposure of a large exposed surface area were also tested. These sources were the 6-kW long-arc xenon lamp and the fluorescent 'blacklight' lamp described below.

Animal study. Animal tests were conducted with the same six chemical agents, under the three laboratory light sources. The animals (hairless mice and miniature swine) are described in the following section.

Screening test

Animals. Hairless mutant mice, Skh:hairless-1 (Forbes & Urbach, 1975) were housed in custom-built stainless-steel irradiation cage units (Forbes & Urbach, 1969). The animals were put into the cages when 6–8 wk old and had free access to laboratory chow and tap-water throughout the period of study. Miniature swine were provided by Federated Medical Resources, Honey Brook, Pa. The breeding stock originated from the Hanford–Labco cross variety (Biology Division, Batelle-Northwest Laboratories, Richland, Washington). Skin features included sparse hair distribution and variable epidermal pigmentation.

Light sources. The 6-kW long-arc xenon high-pressure burner with power supply, igniter and water cooling system (Model RM60) was supplied by the Atlas Electric Co., Chicago, Ill. The proportions of infra-red, visible, UV-A (400–320 nm) and UV-B (320–280 nm) light approximate to those found in a mid-latitude summer sun spectrum. Inherent filtration attenuates the excess infra-red and UV-C (< 280 nm) emitted by the burner (Forbes *et al.* 1976b). The other light source was a bank of four fluorescent 'blacklight' lamps, type F40BL (Westinghouse Electric Corp., Bloomfield, N.J.), with a broad band output in the UV-A region centered over 350 nm (Fig. 1). A sheet of glass, 6 mm thick, was interposed between the source and the skin in order to eliminate the small amount of UV-B emitted by the lamps; this also produced a slight red-shift in the emission peak (Fig. 1).

Light measurement. UV-A flux was monitored with a recalibrated J-221 meter (Ultraviolet Products Inc., San Gabriel, Calif.). UV-B flux was calculated from current flow from a WL767 zirconium phototube (Forbes & Urbach, 1975) or from an R-B sunburning UV meter (Urbach, Berger & Davies, 1975). Within the solar UV-B region these latter devices have response characteristics that closely parallel the action spectrum for 'minimal erythema' of untanned white human skin. The weighted reading is referred to as erythema effective energy of appropriate light sources (Forbes & Urbach, 1975). The spectral output of each lamp was determined by means of a Cary model 14 spectrophotometer with integrating globe radiometer attachment (Forbes, Davies, D'Aloisio & Cole, 1976a).

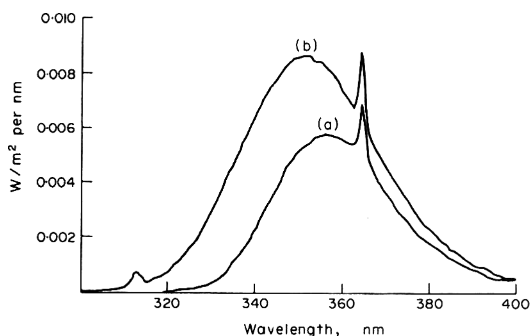


Fig. 1. Emission spectrum of fluorescent blacklight (F40BL) expressed as flux at the spectroradiometer entrance slit, 62 cm from a single lamp, (a) with glass interposed between light and slit and (b) without glass. Integrated UV-A energies are (a) 0·21 and (b) 0·32 W/m^2. At skin level under a bank of ten lamps, the intensity is greater by a factor of 15.

Exposure technique. Twelve mice were treated with each test material. Non-viscous oils and extracts were tested as received; viscous materials, pastes and powders were suspended in methanol or benzene. When necessary, test materials were diluted in order to minimize primary irritant reactions or a 'sun screening' effect. One group of controls was treated with 8-MOP in methanol (0·01%, w/v), and another group with appropriate vehicle only. Each mouse received a single treatment of 20 μl of the test material pipetted onto approximately 2 cm^2 on the back skin. The area to be treated was defined by a wire loop, and care was exercised to ensure that the volume of fluid was spread evenly over the treated area. The more volatile components evaporated, leaving the test material on the treated area of the skin. Six mice treated with each material were exposed to one of the light sources beginning 30 min after treatment with the material. Two miniature swine were similarly treated with each test agent. The animals were lightly sedated with ip sodium pentobarbitone (25–50 mg/kg) in order to ensure even exposure of the treated areas under the light source. The light sources were used to irradiate circular areas of skin. Each area was defined by a 1-cm diameter hole punched in an aluminum foil adhesive tape. The tape masked the skin surrounding the exposed spot (Fig. 2). Test-agent-treated unirradiated skin served as a control for primary irritant reactions. Animals were exposed to the fluorescent blacklight source for 1 hr at an integrated UV-A intensity of 3 W/m^2. Under these conditions, the blacklight lamps did not produce a detectable skin response by themselves. Exposure conditions with the xenon lamp were adjusted to ensure a barely perceptible erythema by 24 hr after irradiation. Consequently, a chemical agent that elicited a response from skin exposure to the blacklight lamp, or one that elicited more than a barely perceptible response to the xenon lamp, could be considered phototoxic.

Animals were exposed to the solar simulator for 40 min at a distance of 1 m. The spectral irradiance was approximately one half solar constant, i.e., the intensity at skin level over the entire spectrum was about half that of noon summer sunlight (Forbes *et al.* 1976b). The intensity of the weighted erythemal energy was 0·1667 W/m^2. The treated animals were examined 4, 24, 48, 72 and 96 hr after exposure.

Since the aim of this test was to yield information pertinent to the use of the test compounds as fragrance materials (as distinct from flavourings, drugs, dyes etc.), the route of administration was limited to topical application.

Test agents. Materials were received with generic name and numerical code as the only identification. Consequently, each one was 'fingerprinted' for our own purposes by means of visible and UV spectrophotometry. In certain cases, additional information was acquired from fluorescence spectroscopy, gas chromatography or infra-red spectroscopy.

Materials found to be phototoxic were further evaluated by means of dilution assay to estimate their relative effectiveness. The test material was diluted in up to 14 steps, beginning with the original material. Each step involved diluting the solution from the previous step with an equal volume of solvent. Thus, photobiologically active compounds could be com-

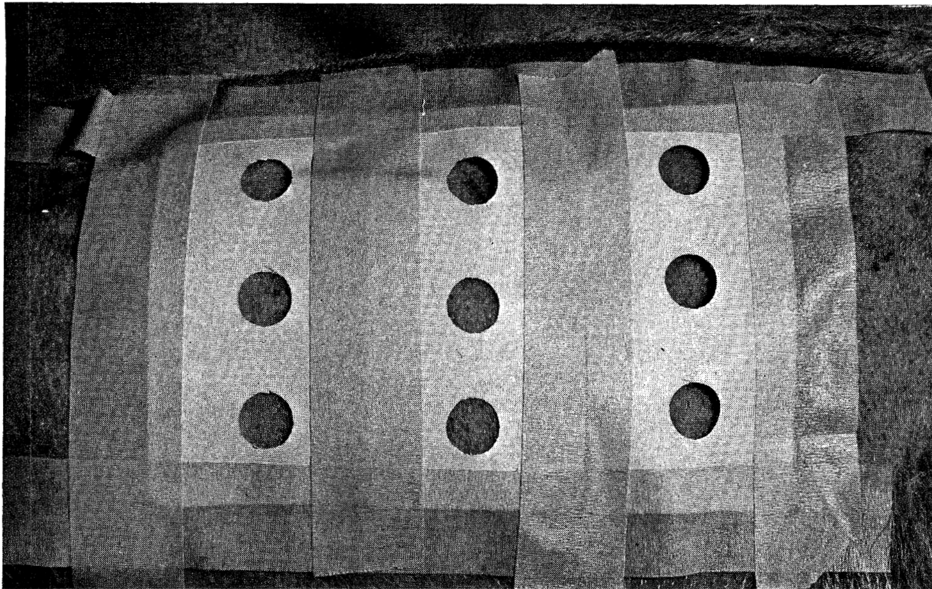
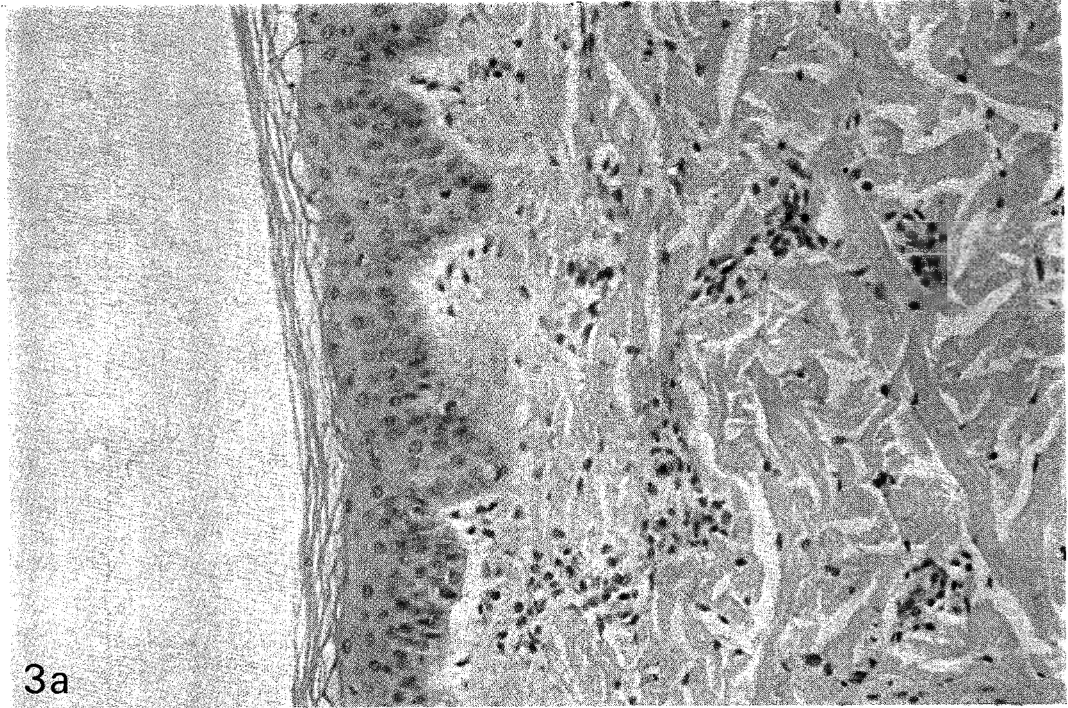


Fig. 2. Skin of miniature swine with mask in place showing circles punched in aluminum foil-backed mask to define the areas of exposure.



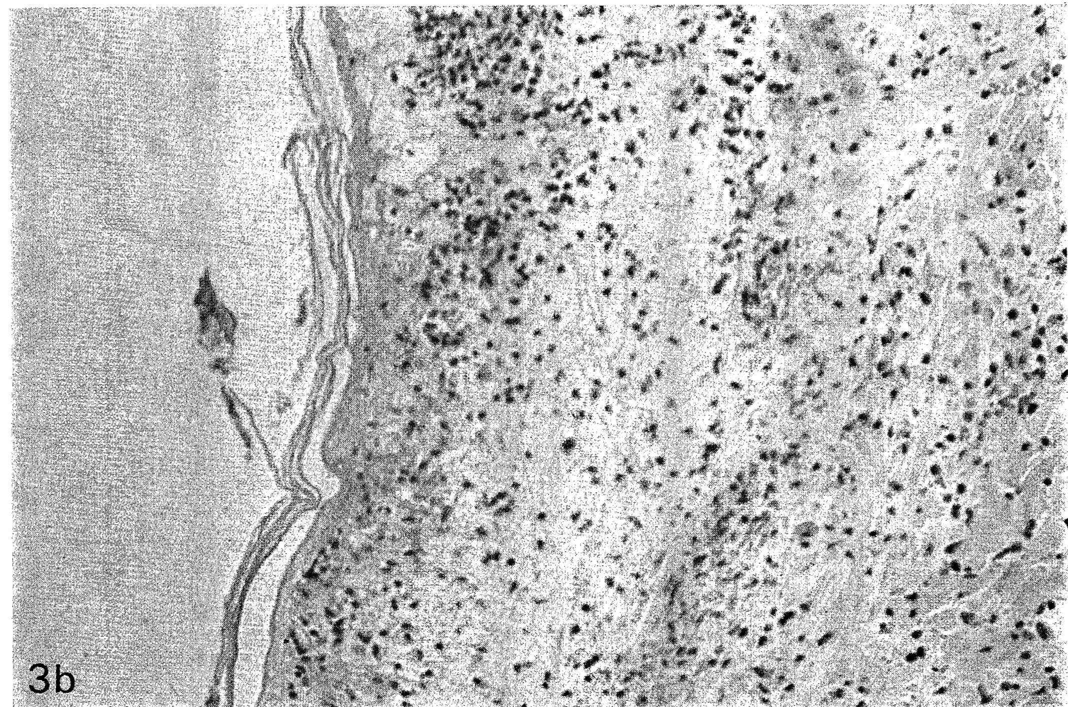


Fig. 3.

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Table 1. *Comparison of phototoxic effects of six compounds on three species under four light sources*

Compound	Species	Sun (30 min)	Compact-arc xenon lamp (2 min WG345 or WG320)	Long-arc xenon lamp (40 min)	F40T12BL Blacklight (60 min)
		Phototoxic response to			
Bergamot A (Expressed)	Human	+	+ (20)	NT	+ (20)
	Pig	NT	+ (20)	+ (20)	+ (20)
	Mouse	NT	+ (10)	+ (10)	+ (10)
Bergamot B (Twice rectified)	Human	–	–	NT	NT
	Pig	NT	–	–	–
	Mouse	NT	–	–	–
Lime A (Distilled)	Human	–	–	NT	NT
	Pig	NT	–	–	–
	Mouse	NT	–	–	–
Lime B (Expressed)	Human	+	+ (30)	NT	+ (30)
	Pig	NT	+ (30)	+ (30)	+ (30)
	Mouse	NT	+ (15)	+ (15)	+ (15)
Sandalwood	Human	–	–	NT	NT
	Pig	NT	–	–	–
	Mouse	NT	–	–	–
8-MOP	Human	+ (0·01)	+ (0·01)	+ (0·01)	+ (0·01)
	Pig	+ (0·01)	+ (0·001)	+ (0·01)	+ (0·001)
	Mouse	NT	+ (0·0003)	+ (0·001)	+ (0·0003)

NT = Not tested

A phototoxic response is indicated by + and an absence of response by –, the figures in brackets indicating the lowest concentrations evaluated that were phototoxic. Oils were diluted to 10% and 8-MOP to 0·0001%.

pared with each other by threshold response at a decimal concentration, or by means of the dilution step, expressed as n where concentration equals $1/2^n$. Results of assaying materials, both singly and in various combinations, will be reported separately.

RESULTS

Preliminary experiment

The sunlight exposure produced a mild but distinct erythema in otherwise untreated areas of human skin. The erythema faded after its peak at 24 hr, and was gone by 72 hr, being replaced by slight tanning. The same response was present in areas pretreated with full-strength sandalwood oil, Bergamot B or Lime A. In contrast, an intense erythema and oedema developed in areas pretreated with Bergamot A, Lime B or an 8-MOP solution. A 2-min exposure to the compact-arc xenon lamp with WG320 filter was sufficient to induce a mild but distinct erythema in previously untreated human skin. The same response required a 40-min exposure to the long-arc lamp under the conditions of use, but there was a very large gain in exposed field size (0·5 m^2 with the long-arc lamp, as against < 1 cm^2 with the compact-arc lamp), allowing all of the test areas on an individual's back, or a large number of pretreated animal sites, to be exposed simultaneously.

With the WG345 filter in place and using the same times for exposure as above, there was no response in areas pretreated with vehicle alone, or with Bergamot B, Lime A or sandalwood oil. In contrast, erythema and oedema developed in areas pretreated with Bergamot A, Lime B or 0·01% 8-MOP. This set of responses was virtually duplicated using the F40BL blacklight lamps (Table 1).

With exposure to the three laboratory light sources, the responses in the skin of miniature swine were generally indistinguishable from those in similarly treated areas of human skin. The histological appearances of pig-skin samples with or without a phototoxic response are contrasted in Fig. 3.

The responses in mouse skin were qualitatively similar, but mouse skin required less of the sensitizing material to elicit symptoms of phototoxicity (Table 1).

Screening test

Exposure to the long-arc xenon lamp resulted in barely perceptible erythema for animals pretreated with vehicle only or with any of the following:

Animal source	Ambergris (74-21)*
	Beeswax (74-133)
	Civet (72-108)
Amaryllidaceae	Narcisse absolute (75-105)
Anacardiaceae	Schinus molle (72-241)
Anonaceae	Ylang oil extra (73-40)
Araceae	Calamus oil (74-54)
Aristolochiaceae	Snakeroot oil (75-129)
Betulaceae	Birch (70-78)
Burseraceae	Myrrh absolute (75-103)
	Oil elemi (74-191)
	Oil linaloe wood (75-91)
	Oil myrrh (72-202)
	Olibanum absolute (74-264)

*Figures in brackets are RIFM reference numbers, and further information on these compounds is available from the Research Institute for Fragrance Materials, Englewood Cliffs, New Jersey 07632, USA.

Castoridae	Castoreum tincture (72-14)
Chemopodiaceae	Chenopodium oil (74-58)
Cistaceae	Cyste absolute (72-118)
Compositae	Armoise oil (74-38)
	Artemisia (wormwood) (74-39)
	Chamomile oil Roman (72-97)
	Costus oil (72-109)
	Deertongue absolute (75-24X)
	Estragon oil (72-134)
	Helichrysum oil (75-69)
	Oil chamomile German (72-98)
	Tansy oil (74-117)
Cupressaceae	Cypress oil (75-21)
Dipterocarpaceae	Gurjon oil (74-205)
Euphorbiaceae	Cascarilla oil (74-57)
Geraniaceae	Geranium Algerian
	Geranium Morocco (74-90)
	Geranium oil (07-2135)
Gramineae	Lemongrass E. I. (71-48)
	Oil palmarosa (72-207)
	Vetiver bourbon (73-39)
Hamamelidaceae	Styrax Asiatic (74-269)
	Styrax oil (74-116)
	Styrax USP Honduras (74-267)
Iridaceae	Orris oil (71-64)
Labiatae	Basil (71-33)
	Cornmint oil (74-63)
	Hysop oil (74-212)
	Lavandin acetylated (71-176)
	Lavandin benzol. absolute (75-85)
	Lavandin oil (73-24)
	Lavender benzol. absolute (75-88)
	Lavender oil (73-25)
	Oil marjoram Spanish (72-185)
	Oil origanum (72-206)
	Patchouly Indonesian (73-31)
	Pennyroyal oil (72-208)
	Sage oil Spanish (72-223)
	Savory oil (75-128)
	Spearmint (74-242)
Lauraceae	Bois de rose acetylated (72-10)
	Camphor oil brown (75-8)
	Camphor oil yellow (74-55)
	Cinnamon bark oil (72-99)
	Cinnamon leaf oil (72-100)
	Laurel leaf oil (73-97)
	Ocotea cymbarum (72-42)
	Oil bois de rose Brazil (73-8)
Leguminosae	Balsam copaiba (74-41)
	Cassia (72-79)
	Fenugreek absolute (75-60)
	Genet absolute (74-135)
	Mimosa absolute (74-105)
	Peru A (72-1)
	Peru B (72-2)
	Tonka absolute (72-245)
Malvaceae	Ambrette seed oil (74-22)
Myrtaceae	Cajeput (74-53)
	Clove leaf oil Madagascar (74-175)
	Clove stem oil (74-61)
	Eucalyptus oil (73-19)
	Oil clove buds (73-16)
	Pimenta berry (75-183)
	Pimento (72-216)
Oleacae	Jasmine absolute (72-174)
Pinacea	Abies cones (72-77)
	Abies needles (72-78)
	Balsam fir oregon (74-165)
	Cade oil rectified (74-129)
	Cedarleaf oil (72-93)
	Cedarwood oil Atlas (74-171)
	Cedarwood oil Texas (75-12)
	Cedarwood Virginia (73-9)
	Fir needle oil Canadian (74-88)
	Juniper (72-175)
	Pinus oil pumilio (75-120)
	Pinus oil sylvestris (75-121)
	Spruce oil (73-35)
	Turpentine oil A (71-85)
	Turpentine oil B (71-86)
Piperaceae	Black pepper (72-79)
Rosaceae	Oil rose absolute Moroccan (72-219)
	Rose absolute French (72-220)
	Rose Bulgarian oil (72-221)
Rutaceae	Amyris (71-29)
	Bergamot B (Bergamot oil twice rectified free of furocumarin 4/012530C)
	Bergamot DLBGC (furocumarin free; X107)
	Eau de brouts absolute (75-42)
	Grapefruit (72-24)
	Lime A (distilled, "psoralen free"; X102)
	Lime A B 2632 exp. rect.
	Limes Persian Florida exp. rect. (72-59 RE)
	Mandarin (72-56)
	Mandarin oil Italian (72-183)
	Oil of lemon California FCF
	Oil lemon distilled (72-240)
	Oil lemon petitgrain (72-242)
	Oil mandarin Italian (74-200)
	Oil neroli bigarde petale (72-244)
	Oil neroli Tunisian (74-226)
	Oil petitgrain Paraguay (73-32)
	Oil tangerine (74-201)
	Orange CP (72-43)
	Petitgrain bigard oil (75-112)
Santalaceae	Sandalwood oil (X101)
Solanaceae	Tobacco leaves absolute (75-135)
Umbelliferae	Angelica seed oil (73-42)
	Anise oil star (74-32)
	Caraway (72-88)
	Carrot seed oil (74-56)
	Celery seed oil (72-95)
	Cuminic aldehyde (72-246)
	Dill weed oil (75-36)
	Fennel oil bitter (75-59)
	Galbanum resinoid (74-265)
Violaceae	Violet leaf absolute (75-IFRA-9)
Zingiberaceae	Ginger (72-156)
	Cardamon oil (72-89)
Zygophyllaceae	Guaicwood oil (73-22)
Reagents	Cyclopentadecanolide (74-77)
	Hexyl salicylate (74-91)
	Methanol

	Methyl nonyl ketone
Mixed or unidentified botanical families	Ale oil (74-163)
	Cognac oil, green (74-62)
	Flouve oil (74-199)
	Foin absolute (75-160)

A similar light exposure produced a phototoxic response in animals pretreated with the following materials:

Rutaceae	Bergamot 1 (expressed; X105)
	Bergamot 2 (expressed; X106)
	Bergamot A (expressed 1970 crop; X104)
	Bergamot expressed (31-66)
	California lemon (72-32)
	Italian lemon (72-61)
	Oil lemon C.P. Greek (72-24)
	Oil lemon Italian (72-250)
	Oil lemon I.C. (72-251)
	Oil lemon Italian (72-249)
	Lime B expressed (X103)
	Oil limes Persian (Florida exp. rect.; 72-59RE)
	Oil limes exp. rect. (Pers.) (72-59A)
	Lime exp. rect. (72-59)
	Rue oil (74-114)
	Rue oil (74-124)
	Bitter orange (72-60)
Umbelliferae	Angelica root oil (74-127)
	Cumin (72-112)
	Oil opopnax (73-67)
Verbenaceae	Verbena oil French (75-141)
Reagents or isolates	Anthracene
	8-Methoxypsoralen (8-MOP)
	5-Methoxypsoralen (5-MOP)

Parallel results were obtained with the blacklight lamp. In mice, erythema was accompanied by oedema and followed by crust formation and eventually sloughing. In miniature swine, the erythema began to fade by 72 hr and was gradually replaced by a mild but persistent tanning. The mouse was the more sensitive of the two species used in this test system (Table 1).

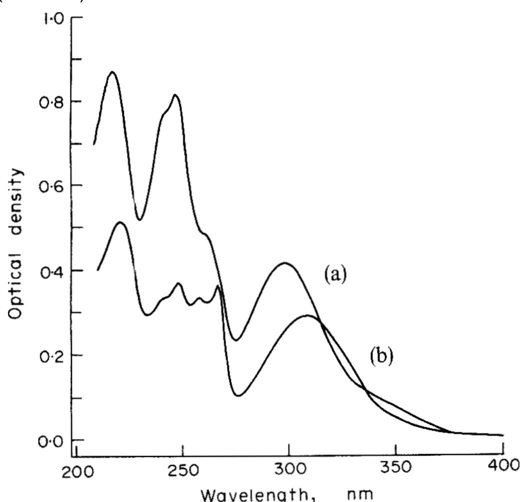

Fig. 4. U.V. absorption spectra of 8- (a) and 5-methoxypsoralen (b).

Figure 4 shows that 8-MOP (also known as methoxsalen, xanthotoxin and ammoidin) was spectrophotometrically similar to 5-methoxypsoralen (5-MOP, bergapten), these compounds having a peak absorption near 300 nm. A characteristic of phototoxic fragrance materials suspended in methanol is the presence of an absorption peak near 312–315 nm (Fig. 5). Suspension in hexane causes about a 5-nm blue-shift in the peak. Spectrophotometric and chromatographic data appear to confirm the presence of 5-MOP in most of the phototoxic materials, but the apparent content does not correlate well with phototoxic activity.

Rue oil and verbena oil (75-141) were phototoxic but neither appeared to have a distinct absorption peak at 312–315 nm (the curve for rue oil is shown in Fig. 6). The signs of phototoxicity induced by anthracene or verbena oil began to disappear by 24 hr; for all other phototoxic fragrance materials, the peak response was at about 48 hr.

DISCUSSION

Of the five fragrance materials tested in sunlight on human subjects, two were readily identified as having phototoxic potential. The same compounds were reactive under the other light sources, indicating that these sources have sufficient activating output and that the photobiological effect could be produced by UV-A alone. The exposed field of the long-arc lamp, although only 1/20 as intense as that of the compact-arc lamp, was much more than 20 times as large, permitting greater efficiency in testing multiple compounds.

The activation spectrum for any particular photosensitizing compound is a function of its absorption spectrum, although in a heterogeneous medium the biological activation spectrum may be affected by chemical or physical interactions (Pathak, 1969). In any case, each photosensitizing compound has a specific, and usually limited, spectral band over which it can be activated. Some of the known sensitizers are activated in the visible light range; a few compounds are activated principally by UV-B. We antici-

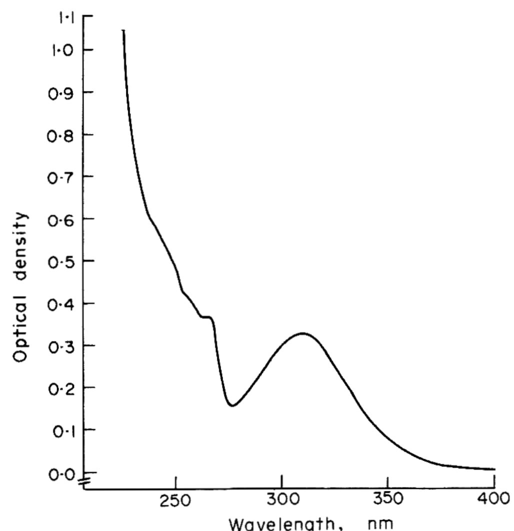

Fig. 5. UV absorption spectrum of bergamot oil.

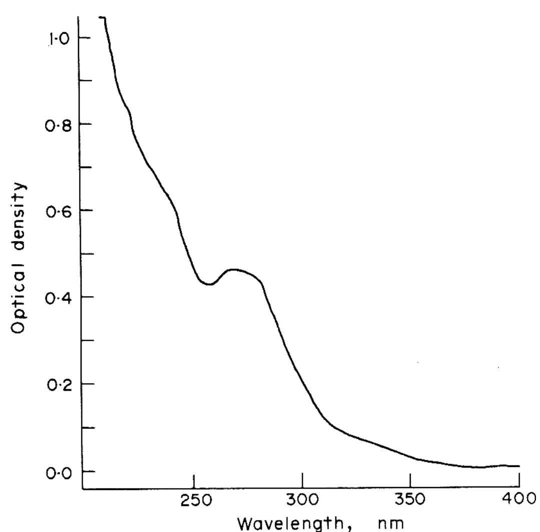

Fig. 6. UV absorption spectrum of rue oil.

pated that most phototoxic components of topically applied materials would be activated by photons in the UV-A region; however, since other possibilities could not be excluded (Gardiner, Dickson, Maclead & Frain-Bell, 1972) we chose to use a simulation of the sea-level sunlight spectrum as the primary test-light source. The fact that the listed compounds reacted under both the xenon arc solar simulator and blacklight sources confirms that the primary activation spectrum of these compounds lies within the UV-A region. Of approximately 160 fragrance material samples tested, 22 were found to be phototoxic.

The results indicate that hairless-mouse skin is a more sensitive test system for phototoxicity than is the skin of miniature swine; the skin of man and miniature swine react similarly to light exposure, both in terms of UV-B and phototoxicity responses. Consequently, we believe that the responses of miniature swine skin can be taken as predictive of human response, while the hairless mouse provides a more sensitive screening system.

All but one of the phototoxic fragrance materials were derived from members of the botanical families Rutaceae or Umbelliferae. The sole exception was verbena oil (75-141), from a member of the Verbenaceae. As noted before, this material lacked the spectral absorption peak at 312–315 nm usually associated with fragrance material phototoxicity and it provoked somewhat unusual phototoxic symptoms.

Psoralens have long been associated with phototoxicity (Urbach, Forbes, Davies & Berger, 1976), and several members of the psoralen family have been described and evaluated (Cieri, 1969; Musajo & Rodighiero, 1962). Analytical gas chromatography provided evidence of 5-MOP in most of the phototoxic fragrance materials reported here. However, the apparent amounts did not correlate well with the levels of phototoxicity. We are examining several hypotheses, including the possibilities that a fragrance raw material may contain several qualitatively different phototoxic components and that other components may either enhance or inhibit the expression of phototoxicity.

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SHORT PAPER

NON-TERATOGENICITY OF D- AND L-GOITRIN IN THE RAT

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Summary—Various levels of D- or L-goitrin were administered to rats in a single oral or intramuscular dose on day 9, 11, 13 or 14 of pregnancy. A study of the foetuses at term revealed no apparent embryopathic effect.

Introduction

Goitrin (L-(−)-5-vinyl-2-oxazolidinethione) is a potent goitrogenic compound which exerts its effect by blocking the iodination of thyroxine precursors (Astwood, Bissell & Hughes, 1945; Clements, 1960). Plants belonging to the genus Brassica contain goitrin, concentrations of 0·12–1·0 g/kg having been reported for turnip roots (Astwood, Greer & Ettlinger, 1949) and 2–4 g/kg for rapeseed meal (Raciszewski, Spencer & Trevoy, 1955), and the occurrence of both goitrin and its precursor progoitrin (2-hydroxy-3-butenyl glucosinolate) in plants has been reviewed (Golberg, 1967). Progoitrin can be hydrolysed to yield goitrin by the action of the thermolabile enzyme myrosin and a variety of intestinal flora of human and rat origin (Greer, 1956 & 1962; Karawya, Wassel & Elmenshawi, 1973; Kjaer, Gmelin & Jensen, 1956). Human exposure to goitrin and progoitrin may occur during the ingestion of raw or cooked food and cows' milk. However, the exposure from cows' milk is considered to be the lesser hazard because the goitrin was shown to be undetectable after a 24-hr storage period (Kreula & Kiesvaara, 1959).

Chemical synthesis of goitrin results in two biologically active optical isomers (Ettlinger, 1950) of equal goitrogenic activity. This activity amounts to 2 and 133% of the potency of propylthiouracil in the rat and in man, respectively (Greer, 1962).

Several chemicals showing antithyroid activity are also teratogens or have been suspected of having a teratogenic effect. These include thiouracil and its derivatives (Bongiovanni, Eberlein, Thomas & Anderson, 1956; Hepner, 1952), iodine taken over a long period (Bernhard, Grubin & Scheller, 1955; Carswell, Kerr & Hutchinson, 1970), radioactive iodine (Fischer, 1951; Hamill, Jarman & Wynne, 1961; Russell, Rose & Starr, 1957) and ethylene thiourea (Khera, 1973). The teratogenicity of goitrin has apparently not been investigated.

Preliminary studies to evaluate the teratogenicity of goitrin and allyl isothiocyanate, another goitrogenic agent present in plants (Golberg, 1967), were conducted in the rat, in our laboratory. The results obtained with allyl isothiocyanate have been published elsewhere (Ruddick, Newsome & Nash, 1976), and the data obtained with goitrin are now reported.

Experimental

Female Wistar rats weighing 175–200 g were paired overnight with proven sires, and the morning on which a sperm-positive vaginal smear was observed was considered to be day 1 of pregnancy. Five or six mated females were randomly assigned to each experimental group. Each isomer of goitrin (Lancaster Synthesis Ltd., England) was dissolved in a vehicle (methanol–0·1 M-NaOH, 35·5:12 v/v, adjusted to pH 7·8) to give a final concentration of 111 mg/ml. The two goitrin isomers were administered individually at various dose levels either orally by intubation on day 9 or 13 of gestation, or intramuscularly on day 11, 13 or 14 of gestation. Appropriate controls were given an equivalent amount of vehicle alone by the same route. The rats were killed on day 22 of gestation, and the foetuses were removed, weighed and examined for external malformations. Two thirds of the live foetuses were studied for skeletal anomalies, and the remainder were examined for visceral changes.

Statistical analyses of intergroup comparisons were performed using Student's t test, and differences were considered significant for $P < 0{\cdot}05$.

Results

No overt signs of maternal toxicity were observed in rats from any of the experimental groups. The mean number of live foetuses/litter in all treatment groups was similar to that in control rats (Table 1). The ratios of foetal deaths to total implants for the various dosing schedules of the two goitrins did not differ statistically from the control values. The ratio for the L-goitrin-control group treated intramuscularly on day 13 of gestation was high because one litter had nine deciduomas and three live foetuses. Dosing with D- or L-goitrin caused no statistically significant reduction in foetal body weight.

The incidences of anomalous foetuses and anomalous litters are also given in Table 1. For all test

Table 1. *Prenatal effects of goitrin following administration on day 9, 11, 13 or 14 of gestation in rats*

Treatment group	Dose (mg/kg)	Day of gestation at dosing	Route of dosing	No. of dams	No. of live foetuses/litter (mean ± SD)	Dead foetuses (%)*	Foetal weight (mean ± SD)	Incidence of anomalies (no. anomalous/total examined)	
								Foetuses	Litters
Control	0	9 & 13	oral	5	13·4 ± 2·2	6	5·2 ± 0·3	11 67	3/5
D-Goitrin	186	9	oral	6	11·0 ± 3·5	4	5·3 ± 0·2	7/66	3 6
D-Goitrin	186	13	oral	5	12·8 ± 1·5	2	5·2 ± 0·3	6/64	2/5
Control	0	9 & 13	oral	6	10·6 ± 2·3	7	4·9 ± 0·4	3/56	2/6
L-Goitrin	25	9	oral	5	11·8 ± 2·4	6	5·0 – 0·4	1/58	1/5
L-Goitrin	25	13	oral	6	10·5 ± 1·6	7	5·2 ± 0·4	7/59	3/6
Control	0	11	im	5	11·6 ± 1·7	3	5·4 ± 0·2	8/58	5/5
D-Goitrin	117	11	im	5	11·6 ± 3·4	0	5·0 ± 0·7	5/59	2/5
Control	0	11	im	6	11·5 ± 2·5	3	5·2 ± 0·2	4/36	2/6
L-Goitrin	100	11	im	6	12·0 ± 1·3	9	4·8 ± 0·2	0/25	0/6
Control	0	13	im	5	9·6 ± 4·8	4	5·6 ± 0·6	9/48	4/5
D-Goitrin	113	13	im	5	12·2 ± 1·1	0	5·2 ± 0·3	10/65	4/5
Control	0	13	im	6	10·3 ± 3·8	21	5·2 ± 0·3	1/31	1/21
L-Goitrin	106	13	im	3	11·3 ± 1·5	6	4·8 ± 0·1	1/31	1/3
Control	0	14	im	4	13·3 ± 1·7	2	5·3 ± 0·5	1/53	1/4
D-Goitrin	112	14	im	5	12·2 ± 1·6	3	5·1 ± 0·1	6/61	4/5
Control	0	14	im	5	12·4 ± 0·9	10	5·1 ± 0·4	3/30	3/5
L-Goitrin	100	14	im	5	11·4 + 1·1	5	5·2 ± 0·4	1/33	1/5

$^{*}\frac{\text{Deciduoma + dead}}{\text{Total implants}} \times 100$

groups these incidences were of similar magnitude to those of the controls. The anomalies were randomly distributed and were those known to occur in the strain of rat used (wavy ribs, extra or 14th rib, and retarded ossification of sternebrae and calvarium).

Discussion and Conclusion

Langer (1966) reported that daily oral dosing with 0·1 mg goitrin/rat for 21 days increased the thyroid weight and radioiodine uptake and decreased thyroid hormone biosynthesis in the treated rats compared with the controls. A single oral dose of 0·1 mg has been shown to inhibit radioiodine uptake by the thyroid 4 hr after treatment (Krusius & Peltola, 1966). The 25–186-mg/kg doses tested for teratogenicity in the present study were 50–372 times higher than the antithyroid dose of 0·1 mg/rat (estimated to be 0·5 mg/kg) reported by Langer (1966) and Krusius & Peltola (1966) and greatly in excess of the levels of goitrin likely to be consumed by man.

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Early Detection of Health Impairment in Occupational Exposure to Health Hazards. Report of a WHO Study Group. *Tech. Rep. Ser. Wld Hlth Org.* 1975, **571.** pp. 80. Sw.fr. 8.00 (available in UK from HMSO).

In 1973, the WHO Expert Committee on Environmental Health Monitoring in Occupational Health recommended that detailed guidelines should be produced, with WHO assistance, for the comprehensive monitoring of workers' health to meet the different needs and resources of industrialized and developing countries.

The present report of the WHO Study Group, which is the first step towards the development of a long-term programme relating to the early detection of occupational impairment of health, provides a critique of the principles underlying health monitoring, as well as guidelines for use in occupational health practice. Future WHO Study Groups are expected to provide guidelines on changes in health parameters among occupationally-exposed workers, including the establishment of maximum permissible limits for occupational exposure.

Early detection has been defined as "the detection of disturbances of homeostatic and compensatory mechanisms while biochemical, morphological and functional changes are still reversible". The report examines the various parameters used so far in the early detection of health impairment due to carbon disulphide, inorganic lead, organophosphorus and organochlorine pesticides and noise.

The report also discusses fundamental aspects of health impairment, health evaluation, inter-individual and intra-individual variability, epidemiological methods in occupational health evaluation and the health effects of psychological factors encountered at work. Finally, recommendations are made for action by WHO, governments and occupational health institutes, and priorities are specified for the long-term WHO programme.

Annual Report 1974. HM Chief Inspector of Factories, Department of Employment. HMSO, London, 1975. pp. xiii + 133. £2.05.

The control of occupational carcinogens was the dominant theme of the work conducted by the Industrial Hygiene Unit during 1974, with vinyl chloride leading the field. The origins and development of the code of practice for vinyl chloride are outlined in the above report, the aim in the UK being to set a practical standard that a diligent industry could achieve. The Unit is now conducting an epidemiological study of workers in the PVC-processing industry, in which vinyl chloride levels are generally well below 10 ppm even without specific control measures, in an attempt to determine whether such low levels are associated with the development of angiosarcoma.

Experimental evidence that the related vinylidene chloride is also carcinogenic came to light in 1974 from studies on rats, and industry is undertaking further work to explore the situation. An initial check on health records revealed no adverse effects in the small number of employees exposed to the vapour during manufacture, and precautions similar to those for vinyl chloride have now been instituted by the manufacturer concerned. Further evidence relating to the carcinogenicity of propane sultone also emerged during the year, as a result of which, discussions were held with the sole UK supplier and a useful exchange of information took place. Surveys of factories using methylene-bis-*o*-chloroaniline continued, and generally satisfactory conditions were reported at all but one firm, for which a Court Order requiring remedial action was considered necessary. Sampling for bis-chloromethyl ether in areas where hydrochloric acid and formaldehyde might come into contact (e.g. in paper and textile processing) showed no evidence of its formation.

A special exercise, in which 361 factories using mineral oil were visited, revealed that although employers were generally aware of the hazards, there was considerably less awareness among employees. The implications of this and other findings are now under consideration. The results of an 11-year study of the health of workers at the sole antimony oxide-manufacturing plant in the UK tended to strengthen the suspicion that exposure in an antimony smelting process was associated with an increased risk of lung cancer, and the workers concerned have been informed by means of a booklet from the Chief Employment Medical Officer. Meanwhile, the study is continuing. Sampling of representative factories handling inorganic arsenic indicated that those engaged in the manufacture of arsenic compounds, wood preservatives and sheep dips suffered the highest potential risk, fairly high dust concentrations frequently being found in the tipping, bagging and weighing operations.

Other work in the industrial hygiene field involved lead, for which the first year of implementation of the code of practice for health precautions proved generally satisfactory. An annex to the code for the lead-battery industry was almost completed during the year, and preparation of a section on biological monitoring was under consideration. A long-term study of occupational exposure to lead is now in progress. During 1974 the number of cases of lead poisoning fell to 36, and the number of employees

who had to be removed from lead exposure due to excessive absorption fell to 154. both totals being well down on those of previous years. The demolition industry was responsible for 16 of the poisoning cases, which arose because of the impracticality of providing local exhaust ventilation and because breathing apparatus was not provided, was unsuitable or was not worn.

In 1974 asbestos exposure was linked with 149 deaths, of which 75 were due to or accelerated by asbestosis, and 139 new asbestosis cases were recorded, reflecting poor conditions in the past. Unsatisfactory conditions in factories and sites led to 14 successful prosecutions. including several of demolition contractors who appeared to be unaware of the need to identify insulation before starting work. A medical and environmental survey of asbestos workers is now nearing completion, and a survey of smaller firms covered by the Asbestos Regulations 1969 revealed that in only 75·8% of cases were asbestos concentrations in the workers' breathing zones below the hygiene standard of 2 fibres/ml. Detailed investigations showed that standards of dust control needed to be improved, particularly in connexion with the machining of asbestos-based components, and follow-up visits are being made to ensure that this advice has been heeded.

During 1974 a survey was initiated of a new polyester/cotton spinning and weaving factory, which presented an opportunity for studying the early effects of cotton dust in order to determine the aetiological factors in byssinosis. The Shirley Institute in Manchester was commissioned to carry out research into methods of reducing dust and fly during winding and beaming processes, which have recently been found to be associated with a high prevalence of the disease. Other specific problems dealt with during the year were an outbreak of arsine poisoning due to leaking cylinders on board a ship, where the arsine concentration was found to be 2000 times the TLV, and a fatal incident involving ethyleneimine exposure under conditions of inadequate ventilation. Concentrations of isocyanates of over four times the TLV were detected in a factory in which a wire at 400°C was used to cut polyurethane foam blocks although, fortunately, the workers have shown no adverse effects as yet.

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BOOK REVIEWS

Effects and Dose-response Relationships of Toxic Metals. Proceedings from an International Meeting Organized by the Subcommittee on the Toxicology of Metals of the Permanent Commission and International Association on Occupational Health, Tokyo, November 18–23, 1974. Edited by G. F. Nordberg. Elsevier Scientific Publishing Company, Amsterdam, 1976. pp. xv + 559. Dfl. 155.00.

This book presents the proceedings of a meeting of an international working group invited by the Subcommittee named above to discuss the toxicology and epidemiology of toxic metals and, more specifically, of the heavy metals, lead, mercury and cadmium. These metals were selected for particular study because of their widespread distribution in the environment and the particularly serious human problems for which their toxic potential may be held responsible. Moreover, each of these metals is likely to occur as a food contaminant to a degree that can make an important contribution to the total body burden.

Various aspects of the toxic effects of heavy metals under conditions of industrial exposure are reviewed in this volume and particular attention is paid to the estimation of a 'dose' in relation to toxic manifestations. The amount absorbed through the skin, lungs or intestine of any given individual is extremely difficult to calculate, particularly in cases of prolonged exposure, and the difficulties involved and the way in which they have been tackled are very well described in the Consensus Report from the meeting. In many respects, this is the part of the book most worth reading and considering in depth. It contains a wealth of concepts and ideas that are clearly relevant to our understanding of the toxicity of heavy metals. These concepts should prove useful in providing a means by which 'threshold' effects may be identified, and one trusts that they may serve also to trigger ideas on how to assess the low levels of intake of these metals resulting from their presence in food.

The contributed articles forming the main part of the publication consider work on the toxicity of heavy metals, covering not only experimental, but also clinical and epidemiological aspects. Most of the data reported have appeared already in other published work and in reviews, but the theme of dose-response relationships is emphasized to a much greater extent here than in most texts. This approach makes this volume considerably more valuable to toxicologists than many other reviews and articles on these topics.

Our Polluted Food: A Survey of the Risks. By J. Lucas. Charles Knight & Company Ltd., London, 1975. pp. xii + 237. £4.95.

The alarmist title of this book leads one to fear yet another polemic from one of the many prophets of doom. It is a relief to find instead a notably moderate, cautious and well-balanced review of the more toxic classes of food contaminants and their possible hazards. A 50-page chapter is devoted to radioactive pollutants in food, reflecting the fact that the author is a lecturer and Deputy Head of the Radiological Protection Service in the University of Manchester. However, he has obviously read widely beyond his own specialist field and discusses with equal facility the problems that may arise from residues of pesticides, polychlorinated biphenyls (PCBs), nitrates, antibiotics, hormones, bacteria, polycyclic hydrocarbons from diesel-engine fumes, zinc, cadmium, mercury and lead. Among these potentially far more dangerous substances, direct food additives and packaging migrants are mentioned only in passing.

The introductory chapter curiously lists only carcinogenesis, mutagenesis, teratogenesis and effects on the central nervous system and liver enzymes as the principal biological effects of chemicals, but it becomes clear from the following chapters that toxic effects on other organs and tissues have not been neglected. For each contaminant, the routes of entry into the environment, the passage through the food chain, the levels in man and the chief biological effects are reviewed, and the actual human intakes are discussed in terms of any acceptable daily intake recommended by FAO/WHO. Wisely, the author refrains from concluding that any substance under discussion is definitely toxic to man at present levels of exposure, although in some cases he suggests that further data are required.

It is noted that the evidence linking food pollution directly with toxic effects in man is extremely limited, being confined mainly to outbreaks of mercury and cadmium poisoning. Possible effects in man can be deduced normally only from animal experiments, and these may have serious limitations owing to interspecies variations in metabolism or tissue sensitivity. More questionably, the author then concludes that only DDT, PCBs and lead present a degree of potential hazard comparable with that of radioactive fission products. Cadmium, mercury, Salmonella, diethylstilboestrol and nitrates are placed in a lower-risk category. Tissue culture is suggested as an alternative to animal studies for revealing the mechanisms of damage and the nature of any interactions that take place at the cellular level, although the difficulties in extrapolating results of such studies to the whole organism are acknowledged.

One disadvantage of the book is that it appears to have been largely completed in late 1971 or early 1972, and was thus already somewhat outdated as a survey of current food-pollution problems at the time of its publication. Notable omissions are the 1973 UK surveys of mercury and cadmium in the diet (*Cited in F.C.T.* 1974, **12**, 139), and even the FAO/WHO 'provisional tolerable weekly intakes' for these metals and for lead (*ibid* 1974, **12**, 407) appear only as addenda to the appropriate chapters. Moreover,

some of the specific gaps in the literature pinpointed by the author, such as the lack of metabolic data on the chlorophenoxyacetic acid pesticides, had already been filled before this book was published (*ibid* 1972, **13**, 475; Gehring *et al. Toxic. appl. Pharmac.* 1973, **26**, 352; Piper *et al. ibid* 1973, **26**, 339). It is also unfortunate that errors have crept into some of the chemical formulae, in particular those on pages 47, 60 and 102, where the aromatic rings have become inexplicably saturated.

However, the book is still of considerable value for its careful appraisal of the possible hazards from the more toxic food pollutants. It should be of interest to a far wider readership than the environmental-science students for whom it was primarily intended.

Of Acceptable Risk: Science and the Determination of Safety. By W. W. Lowrance. William Kaufmann, Inc., Los Altos, Cal., 1976. pp.x + 180. $4.95.

This publication from the United States deals with the rationale of decision taking on problems involving the safety of chemicals, technological processes and environmental changes induced by man. In the past decade 'consumer protection' has become a household phrase, and its promoters can claim a high rate of success in influencing the banning of established products and in hindering the introduction of new ones. No doubt, a sincere intention to lower hazards for consumers has motivated these efforts, but there has been a growing feeling during this period that the price to be paid for absolute safety, achieved only by prohibition, is too high and, of greater importance, that such steps may be to the detriment of the consumer. Another aspect that is increasingly deplored is the slender and often illogical basis of some decisions to ban products. This whole process has escalated to the point where serious questions affecting the economic and political stability of a country may arise.

The subtitle of this volume by Dr. Lowrance of Harvard University is *Science and the Determination of Safety*. Commendably brief and well written, the book also deals with the broader concept of the risk–benefit assessment of products currently produced or within the capability of present-day technology. Unlike Rachel Carson, the author is consistently objective in his approach, as is well illustrated by the final chapter "DDT: An archetypal modern problem". Even in this admirable précis of 30 years of debate on the safety of DDT, the author does not commit himself to a conclusion on the relative benefits and hazards of DDT, but the whole chapter is a vivid evocation of this pressing problem, comparing the differences of opinion that have led, for example, to such divergent decisions as the Environmental Protection Agency's ban on DDT in the USA and the World Health Organization's support for its continued use for the benefit of the majority of the world population.

Preceding chapters, concerned with the identification of problems and the basis and possible consequences of official action, are written, understandably, from the American viewpoint. This in no way detracts from the value of the book for non-Americans, however, since the issues raised are common to many countries, although the decisions reached nationally on such problems as nuclear energy, DDT, air pollutants, contraceptive techniques and drugs vary widely. At first sight such parochial solutions may seem deplorable, but on reflection they may prove to be justified. To the American citizen, the benefits of DDT in the prevention of typhus or malaria would be minimal, but to people living in some tropical countries, for whom the choice might be between a miserable and short life without DDT and a better and more prolonged life with DDT, this insecticide would have to possess far greater toxicity than has been demonstrated for the risk to outweigh the advantages of its use. If decisions were applied universally and were to follow the United States practice of the past few years, the long-term effects could be disastrous. A ban on the use of low-cost pesticides in poor countries, in which 90% of personal income may be spent on food, would accelerate the crisis arising from a situation of too little food for too many people.

Few, however, would dispute Dr. Lowrance's identification of the ultimate goal: "to enjoy the greatest benefit at the lowest risk and cost". Unfortunately, for a given product, any final decision made to this end can be based only on opinion and judgement, owing to our inability to make an adequate quantitative estimate of the risks and the benefits.

Screening Tests in Chemical Carcinogenesis. Proceedings of a Workshop Organized by IARC and the Commission of the European Communities and Held in Brussels, Belgium, 9–12 June 1975. IARC Scientific Publications no. 12. Edited by R. Montesano, H. Bartsch and L. Tomatis. IARC, Lyon, 1976. pp. xx + 666. Sw.fr. 120.00 (available in UK from HMSO).

This publication offers several approaches to the problem of developing short-term tests for the prediction of the carcinogenic potential of chemicals. The IARC organized the workshop in collaboration with the Commission of the European Communities and has published the proceedings as part of its aim to co-ordinate international research and to contribute to the dissemination of authoritative information on different aspects of carcinogenesis.

The first part of the text is devoted to an evaluation of short-term bioassays and the relationship between carcinogenesis and mutagenesis, while the remainder presents various hypotheses concerning the metabolism of carcinogens, carcinogenesis *in vitro*, mutagenesis and DNA repair. Throughout the discussions that follow each paper, interesting and stimulating suggestions arise for those engaged in this type of research, although the broad areas covered by carcinogenesis and mutagenesis do not allow the individual topics to be explored in great depth. This is particularly the case in connexion with the studies now in progress on DNA repair.

As well as being of use to the experimental toxicologist, this book will be welcomed by those concerned with making decisions about the risk–benefit balance of compounds, particularly when material under consideration offers a potential therapeutic advantage or is required for use on a large scale.

Much additional research will be needed before the results of short-term tests can be extrapolated with certainty to predict the carcinogenic risk of a compound to man, but from a consideration of all the methods and hypotheses in this book, it appears that a battery of tests measuring a variety of responses is, at present, the best approach to short-term testing. The range of techniques covered in this extensive review prompts speculation on just how many other tests, so far unreported, are being developed in laboratories all over the world. Although this publication brings the reader up to date with the knowledge at present available, the rapid advances being made in this field will no doubt necessitate a further review of the data within a very few years.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 55*. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1975. pp. vi + 152. DM 39.10.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 57*. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1975. pp. vi + 152. DM 39.10.

In the past 10 years or so, Residue Reviews has become something of an institution, and the breadth of its current terms of reference makes it unlikely that the series will ever run short of subject matter.

The papers in the two volumes under review are concerned almost exclusively with pesticides, the one exception being a brief but enlightening contribution in Volume 55 concerning the presence of polychlorinated biphenyl (PCB) residues in American silos. Apart from giving a factual account of the past use of PCBs as an internal coating for silos and the consequent contamination of silage and hence of the body fat and milk of livestock, this paper provides a striking demonstration of the changing attitudes to toxicity and environmental contamination during the 45 years since PCBs became important commercial chemicals. In view of the current awareness of the toxic properties of PCBs and the problems that they present in terms of environmental pollution, it may seem surprising that, in addition to their use in silo coatings, PCBs were suggested in 1930 as a possible substitute for chicle in chewing gum! Internal coating of silos with PCB, a practice largely confined to the eastern half of the USA, began about 1940, but some 30 years passed before the presence of PCBs in milk from cows fed the contaminated silage was recognized and steps were taken to combat the problem.

A contribution occupying well over half of Volume 55 illustrates well the current complexity and sophistication of routine pesticide analyses. A detailed literature review, terminating in a 12-page list of references, describes the automated processes now available in this field. A liberal use of diagrams supports descriptions of existing systems and of possible future developments, and the prospects for 'official' recognition of automated procedures are discussed. Of particular importance to the safety of workers involved in pesticide manufacture or field application is the monitoring of atmospheric concentrations, and the specific problems of sampling aerosol and vapour-phase pesticides for analysis are considered in the third paper in this volume. Again, a comprehensive review of the literature is provided and clear diagrams complement the descriptions of available methods.

The first paper in Volume 57 is concerned with the inactivation of paraquat and diquat by adsorption on soil clays, a question of obvious importance to the functional efficiency of these herbicides. Of more immediate impact for the environmental toxicologist is an interesting and detailed discussion on the residues of various types of pesticides found in the soils and surface waters of the rich agricultural areas of south-west Ontario. Most of the data available relate to organochlorine insecticides, and it appears that the only two insecticides prevalent in Ontario's Great Lakes are DDT and dieldrin. It is claimed that the urban use of organochlorine insecticides for the control of biting flies has been the major source of insecticidal contamination from the Canadian side of the lakes, although clearly agriculture must shoulder some of the responsibility.

Finally this volume presents a detailed consideration of the widespread repercussions, or secondary effects, of the depletion or complete elimination of a species in an ecosystem as a result of pesticidal action. This particular review is concerned specifically with aquatic systems, but it demonstrates clearly the complex interactions between the various plant and animal species in such systems, and the general principles discussed are equally relevant to terrestrial situations. Studies on the secondary effects of pesticides are difficult not only to design and execute but also to interpret, and opinions differ on the possible magnitude and ecological significance of these effects and on the extent to which they may be self-limiting. While recognizing that effort spent on studies of the primary effects of pesticides may be more successful and of greater practical value, the author of this paper emphasizes the importance of collecting data on these chains of secondary effects in order to increase awareness of the problem's existence and of its potential significance. His own paper is a helpful step in this direction.

*Volume 56 of this series was reviewed in an earlier issue (*Food and Cosmetics Toxicology* 1976, **14**, 493).

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Information Section

ARTICLES OF GENERAL INTEREST

RESOLVING THE CYCLAMATE QUESTION

Apart from the well-aired topic of bladder tumours found in some rats fed a cycalmate–saccharin mixture (Price *et al. Science, N.Y.* 1970, **167**, 1131), much of the concern surrounding the use of cyclamates as food sweeteners has centred on the breakdown of cyclamate to cyclohexylamine (CHA) in many animal species, including man (*Cited in F.C.T.* 1972, **10**, 716; *ibid* 1973, **11**, 326), largely as a result of the activity of the gut flora (*ibid* 1972, **10**, 239). CHA has been shown to release catecholamine stores in the body, and may therefore have adverse cardiovascular effects (*ibid* 1970, **8**, 587). It has also been shown to induce chromosomal aberrations. However, studies of its mutagenic potential have produced conflicting results (*ibid* 1972, **10**, 587 & 876), and we now report some further information on this controversial topic.

Lorke (*Humangenetik* 1973, **18**, 165) treated male mice with 10 g sodium cyclamate daily on 5 successive days, and then mated them each week for 10 weeks with three untreated females. All the females were killed on day 14 of gestation, and the numbers of corpora lutea, implantations and live and dead embryos were counted. No reduction in the fertility index or increase in pre- or post-implantation loss was detected in comparison with a control group, and no signs of toxicity were seen in the males. Lorke & Machemer (*ibid* 1975, **26**, 199) fed 1% saccharin or cyclamate (sodium salts) or 0.11% CHA sulphate to mice of both sexes for at least 10 weeks. No effect on appearance, weight gain or behaviour was observed in any animal, and neither fertility nor foetal loss was affected. There was thus no indication that any of the compounds tested had mutagenic activity in mice. The dosage levels were calculated to be equivalent to about 2000 mg sodium cyclamate or saccharin/kg/day and to 136 mg CHA/kg/day. The same authors (Machemer & Lorke, *Arzneimittel-Forsch.* 1975, **25**, 1889) also reported negative findings in spermatogonial preparations from Chinese hamsters treated orally with two doses of 5 g sodium saccharin/kg or five doses of 2 g sodium cyclamate/kg given in each case at 24-hr intervals.

In a different approach, CHA was introduced into living sheep foetuses *in utero*, and cultures of peripheral blood lymphocytes from foetal blood were harvested after 48 and 68 hours (Turner & Hutchinson, *Mutation Res.* 1974, **26**, 407). Each CHA infusion, in a dose of 50, 100, 200 or 250 mg/kg estimated body weight was maintained for 5 or 18 hours. The percentage of foetal lymphocyte aberrations was increased by CHA treatment, and a relatively high frequency of chromosomal abnormalities was observed. The incidence of aberrations was four or five times greater with the 18-hour than with the 5-hour infusion. It appeared that CHA had an effect on the presynthetic part of the G_1 phase of the cell cycle, and the incidence of chromatid aberrations suggested that the G_2 and late S phases were also affected. A dose-related inhibition of cell growth was observed, but there was no evidence in bone-marrow preparations of any overt damage to cellular organelles, membranes or achromatic apparatus.

In male rats given 50 mg CHA hydrochloride/kg/day by mouth or ip injection daily for 5 days, chromosome analysis of bone-marrow samples taken 1 day after the course of treatment revealed no changes (Dick *et al. ibid* 1974, **26**, 199). There was no increase in the incidence of chromosome breakage, where positive control groups exposed to triethylenemelamine or tris-(2-methyl-1-aziridinyl)phosphine oxide showed a higher percentage of breaks.

Tentative investigations of the chromosomal effects of cyclamate in 12 men and women given the compound over a 3-month period had to be curtailed because of imposed restrictions on the use of cyclamate (Dick *et al. loc.cit.*). In the initial stages of the study, subjects who did not convert cyclamate to CHA and others known to do so received approximately 70 mg cyclamate/kg/day, and chromosome analyses were carried out on blood collected on days 1 and 5 of the study. Total urine samples were collected on days 1–4 and were assayed for cyclamate and CHA. Subjects who did not convert cyclamate to CHA remained non-converters, and three of the four supposed converters eliminated considerable amounts of CHA in their urine, with small quantities appearing on day 1 and amounts as high as 753 mg by day 4. Results of chromosome analyses were all within normal limits, and did not alter from those obtained in the same subject before the experiment, the commonest abnormalities seen at both times being gaps.

No series of mutagenicity studies is complete without the intervention of the fruit fly. Knaap *et al.* (*Mutation Res.* 1973, **21**, 341) treated male fruit flies (*Drosophila melanogaster*) with injections of CHA or *N*-hydroxyCHA 4 days after hatching, or raised them from the first larval instar to the adult stage on media containing the test compound. Treated males were mated and the frequency of sex-linked recessive lethals and II–III translocations was scored in the F_2 generation. No evidence of any mutagenic activity emerged with either compound, although more than 0·2% CHA or 0·1% *N*-hydroxyCHA proved highly toxic to the larvae. Another negative study was reported by Vogel & Chandler (*Experientia* 1974, **30**,

621) who found that the frequency of recessive lethals in male germ cells of *D. melanogaster* showed no increase when the insects were exposed to 5 or 25 mM-sodium cyclamate, 10·1 mM-CHA or 8·8 mM-cyclohexanone oxime.

On balance, therefore, the accumulating evidence seems to favour the view that the mutagenic potential of both cyclamate and its main metabolite, CHA, is unlikely to be significant.

[P. Cooper—BIBRA]

SPECIES VARIATION IN METHYLMERCURY UPTAKE

The attribution of the disaster at Minamata to methylmercury poisoning (Eyl, *Clin. Toxicol.* 1971, **4**, 291) and the demonstration that biological methylation of inorganic mercury was possible in the environment (*Cited in F.C.T.* 1971, **9**, 140) prompted a large number of studies on various aspects of the toxicity of methylmercury. In this review, we aim to examine just one of these aspects—that of species variation in the absorption and distribution of methylmercury. This is important in two respects: to predict the likely effects in man it is necessary to identify a species of experimental animal with a similar pattern of distribution and metabolism for methylmercury, and to predict possible levels of human exposure it is desirable to know the probable extent of methylmercury accumulation in animals or animal products consumed by man. In almost all the studies considered below, tissue distribution of methylmercury was studied after administration of methylmercury chloride (MeHgCl) labelled with ^{203}Hg.

Studies in experimental animals

Guinea-pig. After preliminary studies had shown that the guinea-pig was sensitive to methylmercury intoxication. Iverson *et al.* carried out acute (*Toxic. appl. Pharmac.* 1973, **24**, 545) and subacute (*ibid* 1974, **27**, 60) toxicity studies on this species. After administration of MeHgCl, the ip LD_{50} was 5·5 mg Hg/kg and the oral LD_{50} was 16·5 mg Hg/kg, suggesting that the guinea-pig was more sensitive than the rat and the mouse, but less sensitive than the monkey. After a single oral dose of MeHgCl (1 or 10 mg Hg/kg), Hg concentrations declined from day 1 to day 49 in all tissues except the brain and muscle, in which the highest Hg concentrations were reached around day 7 and thus coincided with the appearance of signs of neurological intoxication.

The distribution of Hg in the tissues of the guinea-pig was similar to that of the mouse but different from that of the rat. In particular, the blood–brain ratio of 0·6:1 for the guinea-pig was closer to that in the mouse, monkey, dog and man than that of 15–20:1 in the rat (Magos & Butler, *Arch. Tox.* 1976, **35**, 25). The concentration of Hg in the tissues was proportional to the dose, but the whole-body half-life of 15 days was independent of the dose.

In the subacute study (Iverson *et al.* 1974, *loc.cit.*), in which MeHgCl was given in oral doses of 0·4–400 μg Hg/kg/day for up to 71 days, no signs of intoxication were observed even though the concentration of Hg in the guinea-pig brain was similar to that at which toxic signs are seen in the cat, dog and monkey. This was attributed to the fact that the half-life of methylmercury is shorter in the guinea-pig than in the other species. The kidney showed the greatest accumulation of Hg in excess of the 1:1 dose-dependency expected. Since 42% of the total kidney Hg was inorganic, it was suggested that the slower excretion of this form of Hg might account for the accumulation. In contrast, the Hg in the muscle, which accounted for the largest total amount of Hg, was present almost entirely as methylmercury.

Cat and man. Hollins *et al.* (*Toxic. appl. Pharmac.* 1975, **33**, 438) suggested that the cat was a suitable experimental animal from which to extrapolate to man, since the 76-day half-life of methylmercury in the body (excluding the hair) was very close to that demonstrated in human studies, a mean of 72 days having been reported by Al-Shahristani & Shihab (*Archs envir. Hlth* 1974, **28**, 342). Both of these are longer than the reported values of 34 days for rats (Magos & Butler, *loc. cit.*) and 8 days for mice (Clarkson *et al. Archs envir. Hlth* 1973, **26**, 173). The half-life for the cat (including hair) was 117 days and was shorter than that in the monkey, cited by Hollins *et al.* (*loc. cit.*) as 150 days. These authors pointed out the highly significant effect of including the hair when measuring the total body burden of Hg.

They also observed a large variation in the rate of elimination of methylmercury between different cats and suggested that individuals within a population might show a large variation in total body burden for a given level of chronic intake. Such variation was observed in man by Al-Shahristani & Shihab (*loc. cit.*) in their study of the hair of 48 Iraqi patients who had consumed seed-grain treated with methylmercury. The half-lives, calculated from the variation in Hg content along the length of hair, did not show a normal distribution: 90% were within the range of 35–100 days, while the remainder fell between 110 and 120 days apart from a single value of 189 days. A more detailed study of the significance of the correlation of exposure to methylmercury with the concentration in the hair has since been published (Giovanoli-Jakubczak & Berg, *Archs envir. Hlth* 1974, **28**, 139). The variation of Hg retention by man was also observed by Skerfving (*Toxicology* 1974, **2**, 3), who reported that in four consumers of methylmercury-contaminated fish, the biological half-life of Hg in the blood cells was between 58 and 87 days while in a fifth it was 164 days.

Hollins *et al.* (*loc. cit.*) state that in both cat and man, overt signs of poisoning develop with methylmercury concentrations in the brain of the order of 10 ppm, but the blood–brain concentration ratios of Hg differ in the two species. For cats they calculate values of 0·78–0·98:1 while for man the accepted values are 0·1–0·2:1, which would explain the fact that the concentrations of Hg in the blood previously reported to evoke neurological signs of intoxication

are approximately ten times higher in the cat than in man.

Rat. Despite the difference in blood–brain ratios for Hg between the rat and other species, most studies of methylmercury metabolism have been carried out in the rat. In one such study, Magos & Butler (*loc. cit.*) showed that the blood–organ ratios 24 hours after repeated daily dosage with MeHgCl were highest for the brain and lowest for the kidney, 70% of the body burden being accounted for by the blood, skin and fur. As in the guinea-pig, the percentage of the dose of Hg in the kidneys and the proportion in inorganic form increased with increasing dose levels. These authors found that the concentration of Hg in the blood was not at all times proportional to the body burden or organ concentration, and urged particular caution in estimating levels in the liver, brain and kidney from that in the blood.

Animals and animal products for consumption

Meat and milk. The difference in tissue distribution between methylmercury and inorganic mercury in laboratory animals is well established, but a direct analogy between these animals and ruminants could not be made without first assessing the effect of the rumen.

In a study of 10-week-old male calves, Ansari *et al.* (*J. Anim. Sci.* 1973, **36,** 415) reported that 7 days after administration of a single oral tracer dose of MeHgCl, the concentration of Hg in all tissues was higher than after a similar dose of $^{203}HgCl_2$. This indicated a greater degree of absorption and tissue retention for methylmercury than for inorganic Hg and showed that the two compounds were not converted into the same chemical form in the rumen. The greatest difference in concentrations was in the muscle, which had a concentration of Hg 600 times greater after ingestion of methylmercury than after ingestion of inorganic Hg. This observation is of obvious importance in the production of beef for human consumption.

In a study of lactating ruminants, Sell & Davison (*J. agric. Fd Chem.* 1975. **23,** 803) observed similar absorption and distribution of Hg in a goat and a cow after intraruminal injection of MeHgCl. The highest concentrations were found in the liver and kidney; levels in the muscle, heart, lung and brain were also fairly high. There was a significant difference in the concentrations in the milk, however, 0·28% of the Hg being secreted in the goat's milk but none in the cow's. On repeated administration of 0·5 mg MeHgCl/kg/day to a goat for 9 days, 0·42% was eliminated in the milk in the first 13 days after the start of treatment, and Hg continued to be present after day 13. In contrast, after administration of $^{203}HgCl_2$ at the same level, only 0·21% had been found in the milk by day 13 and virtually none was detected after that time. The concentration in the milk appeared to be related to the concentration in the blood. The total Hg in the milk over the 36-day experimental period was 1·12 and 0·22% of the dose of MeHgCl and $HgCl_2$, respectively.

All the Hg was found in non-lipid solids of the milk, suggesting an association of Hg with protein as in other biological systems. As in other species, the main pathway for the elimination of Hg was in the faeces irrespective of the source of Hg, but apparent absorption in the goat was over 80% for MeHgCl compared with only about 30% for $HgCl_2$.

Fish and fowl. The well-established ability of marine species to concentrate methylmercury has prompted several studies of chickens fed fish or whale meal as dietary protein supplements. Fish meal is widely used and often has Hg concentrations of less than 0·1 ppm, but whale meal, which would be a valuable feed supplement, has been shown to contain up to 10 ppm (Plummer & Bartlett, *Bull. env. Contam. & Toxicol.* (*U.S.*) 1975, **13,** 324).

In a study of the effect of low dietary concentrations of Hg, March *et al.* (*Poult. Sci.* 1974, **53,** 2181) fed 14-day-old broiler chicks for 4–8 weeks on a diet supplemented with herring meal such that the final dietary levels of Hg were 0·008 or 0·038 ppm. The accumulation of Hg in the breast and leg muscle was considered insignificant, but there was a significant dose-dependent accumulation in the liver, kidney and feather tissues. The concentration in the feathers (up to 0·8 ppm) warrants some concern in view of the practice of processing feathers into meal.

Higher dietary levels of Hg were studied by Plummer & Bartlett (*loc. cit.*) who fed mature hens for 7 weeks on a diet supplemented by whale meal such that the dietary concentration of Hg was 0·95 ppm. Since neither metallic nor inorganic Hg was detected in the whale meal, it was assumed that all the Hg was present as organic Hg. Accumulation of Hg was observed in the flesh, bones, offal and feathers of birds fed this diet, the feathers having the highest concentrations (0·7 ppm). The Hg concentration in the flesh was 0·10–0·18 ppm, compared with 0·01 ppm in the controls fed a mercury-free diet.

In the eggs from these hens, the Hg concentration in the white increased sharply from 0·01 to 0·30 ppm over the first 4 weeks, after which it levelled off. The concentration of Hg in the yolk rose to 0·09 ppm and that in the shells to 0·12 ppm, compared with 0.01 ppm in the eggs of control hens. The authors concluded that the use of whale meals containing such levels of Hg was not advisable.

In another study of the effect of dietary Hg on the Hg concentration in eggs (Sell *et al. J. agric. Fd Chem.* 1974, **22,** 248), 10 ppm MeHgCl (corresponding to about 8 ppm Hg) was added to the diet of 68-week-old hens for 10 days. The concentration of Hg in the eggs reached maxima of 10 ppm in the whites on day 12, and 5 ppm in the yolks on day 19. It was calculated that 55% of the Hg ingested was deposited in the eggs within the 70-day experimental period, and that 80% of that in the eggs was in the white. Further studies on the egg white after ip injection of MeHgCl, which produced a similar distribution to that of orally administered MeHgCl, showed that 78–93% of the Hg was associated with the ovalbumin, which constitutes about 54% of the egg white.

In the mallard duck, high Hg concentrations were found in the oesophageal mucosa, liver and kidney 2 days after oral administration of MeHgCl. At a cellular level, selective concentration of Hg was seen only in the central nervous system (Pass, *J. comp. Path.* 1975, **85,** 1).

Finally, a sobering thought from Japan. Studies of three species of fish that feed on organic matter in

three Japanese rivers, two of which receive waste discharged from Hg mines, showed that the concentration factor (concn Hg in fish/concn Hg in water) in these fish was 10,000–25,000 (Matsunaga, *Nature, Lond.* 1975, **257**, 49). From these factors and from the permissible wet-tissue concentration of Hg, the author calculates that water that is to be fished for species with similar concentrating factors should not contain Hg in excess of 0·000016 ppm for total Hg or 0·00004 ppm for inorganic Hg.

[H.R. Potter—BIBRA]

FLUOROCARBONS NEAR AND FAR

In our last review of the fluorocarbon question, we looked at some of the relationships between inhaled concentrations of fluorocarbons, blood levels of the inhaled compound and the development of adverse effects (Charlesworth, *Fd Cosmet. Toxicol.* 1975, **13,** 572). Although the future of those who consume pressure-dispensed cream topping now seems assured, there is still an element of concern for the inhalers of fluorocarbon, even though it appears to be the abusers who are most at risk.

A recent report by Speizer *et al.* (*New Engl. J. Med.* 1975, **292,** 624) has re-opened this question. Several members of a hospital pathology department engaged in the preparation of frozen sections using an F22 (chlorodifluoromethane) aerosol experienced repeated episodes of palpitations of sufficient severity to make them seek medical attention. Comparison of exercise electrocardiograms or 24-hour dynamic electrocardiograms of the sufferers with results from similar tests made before they joined the department and from tests on staff in other departments showed that the incidence of palpitations was 3·5 times higher in the pathology staff exposed to fluorocarbon. These people were exposed to average levels of 300 ppm F22 in the atmosphere during the 2-minute freezing procedure, but higher peak exposures are likely to have occurred since the aerosol consists of almost 100% F22. The problem of the threshold atmospheric levels of fluorocarbon likely to cause an effect has been investigated in relation to blood levels (*Cited in F.C.T.* 1975, **13,** 572).

Flowers *et al.* (*Archs envir. Hlth* 1975, **30,** 353) have now related exposure levels to the onset of cardiac effects in dogs. Fluorocarbon F11 (trichlorofluoromethane) or F12 (dichlorodifluoromethane) was administered, *via* a pump respirator, to a total of 35 mongrel dogs while the rate and depth of respiration of the anaesthetized animals were controlled. Before the start of the treatment, blood electrolytes, gases and pH were checked and established as normal, after an initial stabilization period on air. During the 10-minute fluorocarbon exposure, cardiac activity was monitored electrically and intra-atrial, atrioventricular nodal and His–Purkinje conduction times were assessed from the data. Both arterial and venous blood samples (2 ml in each case) were taken at 3-minute intervals for measurement of fluorocarbon content.

With concentrations of F11 in the inhaled air below 15%, no change in heart rate beyond a slight transient sinus slowing was observed, but 15–17·8% F11 caused reversible sinus bradycardia in half of the animals, and sinus bradycardia terminating in electrical asystole in the rest. Levels of 18–21·5% produced the same general pattern of disturbances in rhythm and conduction and caused death in 15 out of 22 animals. All animals exposed to F11 at levels of 21·5% or more died. Survivors and non-survivors of exposure to 15–17·8% F11 were compared for F11 concentrations in venous and aortic blood and for arterial pH, partial pressures of oxygen and carbon dioxide and base excess, but there were no significant differences. Similar results were obtained for the group exposed to 18–21·5% F11, with the exception of the 4-minute sample, in which higher F11 levels were found in non-survivors. There appeared to be a narrow boundary between the onset of reversible symptoms (15% or 150,000 ppm) and the first lethal concentration (15·5%). With F12, 10-minute exposure to concentrations of over 80%, giving blood levels of fluorocarbon considerably in excess of those achieved with F11, could be administered before cardiac abnormalities occurred. A level of 21·5% (215,000 ppm) F11 was the lowest that killed all the animals.

Peak levels of F11 measured in a beauty-salon over a period of months did not exceed 310 ppm—well below the threshold of 150,000 ppm. On the other hand, experiments with plastic bags filled deliberately with propellant showed that a 'sniffer' might be exposed to 350,000–400,000 ppm F11—levels well in excess of that found consistently to cause death in this study.

F11 has been identified by another group of workers as the most toxic of a series of 15 propellants studied. The first five papers published by this group have been reviewed in detail (Charlesworth, *Fd Cosmet. Toxicol.* 1975, **13,** 393). Of the second five papers in the series, two summarize and classify the results of the first eight (Aviado, *Toxicology* 1975, **3,** 311 & 321). Papers VI and VII in the series (Doherty & Aviado, *ibid* 1975, **3,** 213; Watanabe & Aviado, *ibid* 1975, **3,** 225) deal with the influences of pulmonary and cardiac lesions on the effect of fluorocarbons in rats, and paper VIII (Aviado & Smith, *ibid* 1975, **3,** 241) covers the effects of fluorocarbons on respiration and circulation in primates. A complete summary of these and other data has been published (Aviado, in *Progress in Drug Research,* Vol. 18, edited by E. Jucker; Birkhäuser Verlag, Basel, 1974, p. 365) in a more useful form, using F11 as a reference compound for its less toxic stablemates.

Perhaps not surprisingly, this group found, in studies VI and VII, that existing pulmonary or cardiac lesions enhanced the toxic effects of the propellants. This type of effect was suspected by Taylor & Drew (*Toxic. appl. Pharmac.* 1975, **32,** 177) and was investigated by them, using a strain of Syrian hamster (BIO 82·62) in which an inherited cardiomyopathy

leads to the development of frank congestive heart failure between the ages of 240 and 270 days. Groups of 16 hamsters, consisting in each case of four 150- and four 240-day-old cardiomyopathic (CMP) hamsters and four 150- and four 240-day-old random-bred hamsters, were exposed to room air, to 7·5% nitrogen in room air or to 7·5% F11 in room air for 4 hours. A similar group of 17 animals was exposed to 2% F11 for 4 hours and a group of four normal hamsters was exposed to 10% F11. F11 levels during exposure were monitored, and the animals were watched to see when death occurred. Deaths among random-bred hamsters occurred only during exposure to 10% F11. The young CMP hamsters survived all F11 exposures, but the four 240-day-old CMP hamsters died during the first 30 minutes of exposure to 7·5% F11 and were found at autopsy to have grossly dilated hearts. Four of a group of five 240-day-old CMP hamsters died after 48 minutes of exposure to 2% F11, and the survivor died 2 days later.

The onset of cardiac arrhythmia was monitored in groups of CMP and normal hamsters during a 5-minute exposure to 2·5, 5·0, 7·5 or 10% F11. The highest exposure level caused arrhythmias in both CMP and control animals, but while this effect was observed with levels as low as 2·5% F11 in CMP animals, no cardiac abnormalities were seen in control animals exposed to levels below 10%. The authors point out that the exact biochemical and genetic character of congenital hamster cardiomyopathy is not known, and that there is no clear correlation between human heart disease and this syndrome. However, these findings do suggest that hamsters in which physiological reserves have been depleted by cardiomyopathy are at greater risk than normal animals.

These authors have also been concerned with studies on F11 and F12 in rabbits. Taylor (*Archs envir. Hlth* 1975, **30,** 349) found that a state of hypoxia accentuated the ability of fluorocarbon to induce arrhythmia in this species. Thus, all rabbits exposed for 4 minutes to an atmosphere containing 7% oxygen and 15% F11 developed cardiac arrhythmia, although this effect was induced neither by 15% F11 in room air (19–21% oxygen) nor by a 7% oxygen apmosphere free of F11. Taylor & Drew (*J. Pharmac. exp. Ther.* 1975, **192,** 129) also reported that acute exposure of rabbits to 10 or 20% F12 reduced cardiac output, depressed the maximum rate of change of left ventricular (LV) pressure and caused a slight decline in LV systolic pressure, without affecting heart rate or LV end-diastolic pressure or causing arrhythmia. The effects were not transient since they were more marked at the end of a 30-minute exposure period than after 1 minute, but in each case recovery was complete within 3 minutes of cessation of exposure. The acute effects of F12 were not modified by previous chronic intermittent exposure to 10% F12. The findings of this study, including the results of F12 exposure in animals pretreated with autonomic blockers, reinforced the view that F12 acts by depressing myocardial activity (*Cited in F.C.T.* 1974, **12,** 553) rather than by modifying autonomic reflexes. The fact that no tolerance or adaptation developed on repeated exposure may be attributable, at least in part, to the rapid elimination of fluorocarbon from body tissues. Investigation of the elimination of F11, admittedly more volatile than F12, showed it to be very rapid (*ibid* 1975, **13,** 573).

Niazi & Chiou (*J. pharm. Soc.* 1975, **64,** 763), however, have suggested that F11 has a longer biological half-life than was previously thought and undergoes extensive distribution in the body. This conclusion was based on a study in which F11 in polyethylene glycol 400 was administered iv, since inhalation can lead to large variations in uptake by individual animals. Blood concentrations of F11 following administration of single or multiple doses of this fluorocarbon to unanaesthetized dogs were characterized by a triexponential decay and could be rationalized by a three-compartment open model with average half-lives of 3·2, 16 and 93 minutes for the disposition phases. A computer analysis following a single dose of F11 showed that a period of about 2 hours was required to achieve pseudodistribution equilibrium, after which more than 90% of the dose remaining in the body was retained in the tissue compartments. Thus the accumulation following multiple exposures could result in much higher tissue levels than might be anticipated from the blood level.

Some interesting *in vitro* work has been carried out with F11 (Wolf *et al. Biochem. Soc. Trans.* 1975, **3,** 175). On the basis of its physical similarity to carbon tetrachloride (CCl_4), and the finding of spectral interactions similar to those of CCl_4 with rat-liver cytochrome *P*-450, it was suggested that F11 might be a substrate for the hepatic mixed-function oxidase system. Rat-liver microsomal fractions were incubated anaerobically with 10 mM-F11 at 37°C for up to 1 hour, after which the suspension was extracted with *n*-heptane after cooling, and the extract was examined by gas chromatography. A peak with a shorter retention time than that of F11 was recorded and was identical to that of dichlorofluoromethane. Incubation with various cell fractions of rat-liver homogenates indicated that dehalogenating activity was concentrated mainly in the microsomal fraction. The rate of formation was much slower than that reported for the formation of chloroform from CCl_4. Metabolite formation was enhanced by phenobarbitone pretreatment, and was reduced by pretreatment with mixed-function oxidase inhibitors such as carbon monoxide, SKF 525A and metyrapone. The presence of air, oxygen or CCl_4 also had a marked inhibitory effect.

Another aspect of the metabolism of fluorocarbons is their potential for the release of fluoride, although the stability of the C–F bond is regarded as one reason why the fluorocarbons are less toxic than other halocarbons to higher animals. Dilley *et al.* (*Toxic. appl. Pharmac.* 1974, **27,** 582) investigated fluoride excretion in rats exposed by inhalation to five fluoroalkenes and one fluoroalkane. The compounds were given for 30 minutes at the following concentrations: 2600 ppm hexafluoropropene, 3500 ppm tetrafluoroethylene, 3000 ppm trifluoroethylene, 2200 ppm vinylidene fluoride, 3000 ppm vinyl fluoride and 3000 ppm hexafluoroethane. Groups of 15 animals were exposed to each fluorocarbon, after which five from each group were killed serially for histopathological examination and ten were returned to their individual metabolism cages for 14 days. Animals were given

food and fluoride-free water *ad lib.* A control group kept in metabolism cages for 14 days was used to supply base-line data. After collection of 24-hour urine samples, fluoride concentration was measured immediately. Glucose, protein, occult blood and pH were estimated as indicators of any toxicity, and sodium and potassium were determined in each sample by flame photometry.

A significant increase in urinary fluoride concentration was seen on day 2 in animals treated with hexafluoropropene or vinylidene fluoride. A second significant increase was seen in all groups on days 4–6. Tetrafluoroethylene caused a third significant increase on days 13–14. Diuresis was observed in all animals except those exposed to vinylidene fluoride, and was most pronounced with hexafluoropropene. Sodium excretion remained unchanged but potassium excretion was elevated in all groups except that exposed to hexafluoroethane. Glycosuria was observed in the hexafluoropropene and tetrafluoroethylene groups on days 1–3 and occult blood was found in the former group. The hexafluoropropene group was the only one to show histological changes when examined microscopically. There was marked necrosis of the proximal tubules with dilation, extensive intraluminal slough and diffuse cytoplasmic eosinophilia. By days 3–4, tubular regeneration was well underway, and by day 7 it was almost complete. Gross pathological changes were observed in the kidneys of all groups, with hyperaemia of the medulla and a pale whitish band in the cortex near the cortico-medullary junction.

These findings support the hypothesis that fluoroalkanes are biodegradable. Fluoride is known to impair the function of the proximal tubules, and since this is the probable site of potassium excretion, this impairment may account for the increased levels of urinary potassium in the treated animals. Although the increase in potassium loss was small, it could lead in the long term to potassium depletion. An unexpected finding was the cyclic nature of fluoride excretion, with peaks at days 4–6 and 13–14.

These last two papers are important in their implication that fluorocarbons can be dehalogenated in animal tissues. At the moment, however, the breakdown of fluorocarbons is receiving attention on a far greater scale. As a result of investigations following 'sudden sniffing deaths', the thresholds and mechanisms for cardiac sensitization in animals and man have been gradually elucidated, but now a more sinister spectre has been evoked. Decisions have already been made to ban the use of fluorocarbon aerosols in some American States with effect from 1977, and other States may follow suit, not because of the fear of possible cardiovascular reactions, but because of a potential environmental hazard. Fluorocarbons have been alleged to accumulate in the upper atmosphere and interact with the ozone layer, causing a depletion of this protective layer and thereby increasing the amount of ultraviolet radiation reaching the earth's surface. Ultraviolet irradiation has been implicated in the aetiology of some skin cancers (*Cited in F.C.T.* 1974, **12**, 593), but inevitably there are wide differences of opinion as to the possible significance of fluorocarbons in this connexion (*Lancet* 1975, *II*, 647; Gribbin, *New Scient.* 2 October 1975, p. 12; Jones, *ibid* p. 14; Rowland, *ibid* p. 8; Sherwood, *ibid* p. 7). Nevertheless it seems that the fate of fluorocarbon propellants hangs on a potential hazard far removed from the cardiovascular effects over which scientists have argued for so long.

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SHORT-TERM CARCINOGENICITY TESTS: YOU PAYS YOUR MONEY....

A recent review on the search for appropriate short-term tests for carcinogenicity (Stich *et al. Can. J. Genet. Cytol.* 1975, **17**, 471) compared the effort of those engaged in this task to the tribulations of Sisyphus, the mythical king condemned for ever to roll a boulder of marble to the summit of a mountain only to see it roll down again. While this may be an over-pessimistic view, the development of such tests is certainly no light task since, in the absence of an adequate understanding of the molecular events involved, the validity of any promising test can be established only by demonstrating for a large number of compounds a strong correlation between the results obtained with the test in question and the results of conventional long-term carcinogenicity studies.

Of the short-term procedures currently under consideration, the one that has been assessed most comprehensively is the Salmonella/microsome test (*Cited in F.C.T.* 1975, **13**, 465; *ibid* 1976, **14**, 353) developed by Dr. Bruce Ames and his colleagues, who have compared the results obtained on over 300 chemicals with the carcinogenic potential indicated by conventional animal studies (McCann *et al. Proc. natn. Acad. Sci. U.S.A.* 1975, **72**, 5135). The correlation demonstrated between the two types of test is remarkably good, considering the enormous differences between bacteria and mammals, and while there are major difficulties about accepting the Ames test as an isolated screening procedure for carcinogenic agents (*Cited in F.C.T.* 1976, **14**, 353), it could play a useful part in a comprehensive screening programme.

The choice of possible supplementary tests is relatively wide, at least in theory. The various procedures utilizing other strains of bacteria are likely to suffer from the same disadvantages as the Ames test, but there are also tests based on fungi, Drosophila and a variety of mammalian cells. Prominent among the fungal tests are those involving yeasts (Mortimer & Manney, in *Chemical Mutagens. Principles and Methods for their Detection*, edited by A. Hollaender; Plenum Press, New York, 1971; vol. 1, p. 289) and *Neurospora crassa* (de Serres & Malling, *ibid* vol. 2, p. 311). While these techniques have, as yet, been subjected to only limited testing, it seems likely that if a large number of compounds were screened and suitable drug-metabolizing systems were added to the cultures along with the test chemical, procedures in-

volving yeast cultures could prove as useful as the Ames test. It is doubtful, however, whether they would offer any real advantages, despite the fact that the genetic material in yeasts occurs in a form similar to that found in mammals, while in bacteria its form is markedly different.

At first sight, tests in the fruit fly, Drosophila, look more promising (Abrahamson & Lewis, *ibid* vol. 2, p. 461). Mutations are readily induced in these insects and both dominant and recessive mutations can be demonstrated (Sobels, *Mutation Res.* 1974, **26,** 277). Moreover, these insects possess a drug-metabolizing system which could convert pre-carcinogens to an active form (Casida, in *Microsomes and Drug Oxidations*, edited by J. R. Gillette *et al.*; Academic Press, New York, 1969, p. 517). A short series of tests has demonstrated the induction of mutagenic effects in Drosophila by carcinogens (E. Vogel, Proceedings of a Meeting held at The Royal Society, London, 27 and 28 May 1976) but, as with the yeasts, the small number of compounds tested makes it unwise to draw any conclusions.

Certain practical advantages are offered by mammalian cell systems, based either on tissue-fractionation or on tissue-culture techniques. In the former group, the biphenyl 2-hydroxylase test (McPherson *et al. Nature, Lond.* 1974, **252,** 488) and the endoplasmic-reticulum degranulation test (Rabin *et al.* in *Effects of Drugs on Cellular Control Mechanisms*, edited by B. R. Rabin and R. B. Freedman, p. 27, Macmillan Press Ltd., 1972) have each given positive results with a number of known carcinogens, but further validation and perhaps improvements in technique would be required before either could be used in the screening of compounds for potential carcinogenicity.

We are left, therefore, with several systems based on mammalian tissue culture as possible partners for the Ames bacterial test. The structure of the nuclear DNA in these test systems is similar to that in organs in which cancer may develop in the live animal, and there is evidence that the induction of DNA damage and repair is a characteristic effect of carcinogens (Trosko & Chu, *Adv. Cancer Res.* 1975, **21,** 391). The possibility of a short-term test for carcinogenicity based on the detection of DNA repair was studied by San & Stich (*Int. J. Cancer* 1975, **16,** 284), who demonstrated, for about 60 compounds, an excellent correlation between *in vivo* carcinogenic activity and unscheduled DNA synthesis (usually regarded as evidence of repair in response to DNA damage) in cultured human fibroblasts.

Another system worth exploring involves the use of tissue cultures to demonstrate the growth inhibition and increase in nuclear size that have been known for many years to result *in vivo* from the short-term administration of many carcinogens. A system utilizing these properties is being developed at BIBRA and results will be published shortly.

Yet a third possibility, described by DiPaolo *et al.* (*Nature, Lond.* 1972, **235,** 278), utilizes the changed growth characteristics (transformation) of cells in culture after treatment with a carcinogen. These changes are also characteristic of cells taken from cancer tissue but not of those from normal tissue and, furthermore, such transformed cells will sometimes produce cancer when injected into animals unless they are killed by immunological reaction. So far, a fair correlation has been demonstrated between induction of transformation and known carcinogenic activity.

Finally, considerable interest has been evoked by the so-called 'micronucleus test' (Schmid, *Mutation Res.* 1975, **31,** 9), which is based on the fact that abnormal nuclei produced by a test compound in young, rapidly dividing, red blood cells persist as nuclear fragments in the maturing cells. Unlike most of the proposed short-term tests, this is an *in vivo* procedure and could thus be used to monitor possible carcinogenic metabolites. It has been tried with success on some carcinogens, but would require much more testing before it could become a serious contender for a place in any short-term screening programme.

The choice is wide and will probably grow wider. At present the Ames test is the clear favourite, not only because of its simplicity but because of the relatively extensive assessment to which it has been subjected. For the essential support procedures, any of the four mammalian-cell systems mentioned above could be used with some degree of confidence; while the Stich test may have the edge on the others, an additional mammalian system would be required because of the classes of carcinogens to which the Stich test appears to be unresponsive. At the moment, however, no battery of tests is likely to replace the long-term test in mammals. Apart from the major problem of detecting weak carcinogens, the question remains as to whether false positive results may result from damage at cell sites other than the nucleus. Such false results may be of small moment to those concerned solely with the sensational aspect of this type of research, but they could deprive the public of some compounds that make a useful contribution to the quality of life.

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Fd. Cosmet. Toxicol. Vol. 15, pp. 77-88. Pergamon Press 1977. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

3095. The trial, error and metabolic fate of amaranth

Pritchard, A. B., Holmes, P. A. & Kirschman, J. C. (1976). The fate of FD & C Red No. 2 and its metabolite, naphthionic acid, after different routes of administration in the rat. *Toxic. appl. Pharmac.* **35,** 1.

Conflicting results of feeding trials with the food colouring amaranth (FD & C Red No. 2) have been variously attributed to defects in experimental design, in execution and in interpretation. This emphasis on human error has tended to overshadow the effect of variations in the ability of a given strain of laboratory animal to metabolize the colouring under a range of experimental conditions. For many years, metabolic investigations have shown little advance on the early findings (*Cited in F.C.T.* 1963, **1,** 103), which demonstrated the azo-reduction of amaranth by the gut microflora, the products being naphthionic acid (1-aminonaphthalene-4-sulphonic acid; NA) and 1-amino-2-naphthol-3,6-disulphonic acid. Only recently have the authors cited above extended this work by monitoring the fate of NA after its administration to rats by several routes.

Serum-NA levels were monitored after administration of a single dose of 200 mg amaranth/kg by intubation in water to groups of fasted or fed Sprague-Dawley rats. Concentrations rose to a peak after 2 hr, the maximum serum levels among fasted rats being approximately twice those found in fed animals. Comparable peaks were recorded after similar dosage of fasted and fed animals with 100 mg NA/kg, but thereafter concentrations declined, apart from a period between 5 and 8 hr after dosing when serum levels remained constant in fed rats. Urine and faecal analyses showed that in fasted and fed animals, respectively, 63 and 84% of the oral dose of NA was excreted in 72 hr, while the corresponding proportions of amaranth recovered as NA in this period were 69 and 53%, respectively. The pattern of excretion of both compounds was similar in fasted and fed rats.

When three groups of fasted rats were given oral doses of 2, 20 or 200 mg amaranth/kg, the urinary NA concentration showed a dose-response relationship. Repeated daily administration of amaranth for 5 days, either by intubation in aqueous solution in doses of 20 or 200 mg/kg or by addition to the drinking-water at levels of 0·01, 0·1 or 1 g/litre, resulted in a cyclic pattern of serum-NA concentrations. However, there was no evidence of NA accumulation over this period. In contrast, administration of amaranth in the diet at levels of 0·2, 0·02 or 0·002% for 2·5 wk did not produce this fluctuating pattern of serum NA concentrations; the serum levels were dose-dependent, but appeared to remain constant over the period of administration.

These experiments showed that repeated oral dosing and consumption of amaranth in drinking-water produced NA concentration patterns in the serum quite different from those observed after ingestion of the dye in food. Although the three modes of administration gave rise to approximately the same maximum serum levels, only consumption in food resulted in a prolonged exposure to this maximum. The authors suggest that these findings should be of interest in connexion with the interpretation of currently available toxicity data on amaranth.

FLAVOURINGS, SOLVENTS AND SWEETENERS

3096. More metabolites of *d*-limonene

Regan, J. W. & Bjeldanes, L. F. (1976). Metabolism of (+)-limonene in rats. *J. agric. Fd Chem.* **24,** 377.

d-Limonene (*p*-mentha-1,8-diene) is a major component of the essential oils of many fruits and spices, and both the compound and the oils are used extensively in the food and cosmetics industries.

In the study cited above, ten metabolites were differentiated in the urine of rats fed 40 mg *d*-limonene/kg/day for 10 days. Seven of these metabolites were identified as the α- and β-hydroxy isomers of *p*-mentha-2,8-dien-1-ol and of *p*-mentha-1,8-dien-6-ol (carveol), *p*-menth-1-ene-8,9-diol (uroterpenol), *p*-mentha-1,8-dien-7-ol (perillyl alcohol) and 4-isopropenyl-1-cyclohexene-1-carboxylic acid (perillic acid). Of these, uroterpenol and perillic acid were identified in previous studies in both the rat and the rabbit (*Cited in F.C.T.* 1975, **13,** 474), but 8,9-dihydroxyperillic acid, which was also identified in the earlier studies, was not found in the present study.

Pathways proposed for the metabolism of *d*-limonene involve consecutive allylic oxidations and direct oxidations of the double bonds through epoxide intermediates.

3097. Acetaldehyde and the rat foetus

Kesäniemi, Y. A. & Sippel, H. W. (1975). Placental and foetal metabolism of acetaldehyde in rat. I. Contents of ethanol and acetaldehyde in placenta and foetus of the pregnant rat during ethanol oxidation. *Acta pharmac. tox.* **37,** 43.

Sippel, H. W. & Kesäniemi, Y. A. (1975). Placental and foetal metabolism of acetaldehyde in rat. II.

Studies on metabolism of acetaldehyde in the isolated placenta and foetus. *Acta pharmac. tox.* **37**, 49.

Although the ability of ethanol to inhibit uterine contractions has been used to prevent premature labour, the effects of ethanol treatment on the foetus have still to be elucidated. Ethanol is metabolized in man and animals to acetaldehyde, a well-documented nerve poison, and the acetaldehyde concentration in the peripheral blood has been shown to be higher in pregnant than in non-pregnant ethanol-treated rats (*Cited in F.C.T.* 1975, **13**, 669). The two studies cited above, in which foetal acetaldehyde levels in similarly treated rats were investigated, are therefore of particular interest.

Placentae or foetuses were removed from pregnant rats 4–5 days from term and 25 min after the animals had been given a 2-g/kg ip injection of ethanol. A sample of maternal blood was taken from the aorta at the same time. Ethanol and acetaldehyde levels in the whole foetus, whole placenta and maternal blood were determined chromatographically, and it was found that while the ethanol levels were all approximately the same, in agreement with previous studies, the acetaldehyde concentrations ranged from a maximum in the maternal blood to an undetectable level in the intact foetus. The acetaldehyde concentration of the placenta was some 25% of the level present in the maternal blood. Acetaldehyde, in view of its greater lipid solubility and volatility, would be expected to have a higher membrane permeability than ethanol, and it was suggested therefore that acetaldehyde was largely oxidized during its transfer across the placenta.

Quantitative enzyme data generated in a series of *in vitro* experiments substantiated this assumption. The aldehyde dehydrogenase activity of the placental homogenates was shown to be about 8% of the maternal liver activity, more than sufficient to prevent exogenous acetaldehyde entering the foetus. Should any trace of acetaldehyde cross the placenta, however, it would be oxidized rapidly by the foetal liver, homogenates of which had an acetaldehyde-oxidizing capacity of roughly half of that of the maternal liver. Foetal alcohol-dehydrogenase activity, on the other hand, has been reported to be very low.

3098. Alcohol and the foetal brain

Mann, L. I., Bhakthavathsalan, A., Liu, M. & Makowski, P. (1975). Placental transport of alcohol and its effect on maternal and fetal acid–base balance. *Am. J. Obstet. Gynec.* **122**, 837.

Mann, L. I., Bhakthavathsalan, A., Liu, M. & Makowski, P. (1975). Effect of alcohol on fetal cerebral function and metabolism. *Am. J. Obstet. Gynec.* **122**, 845.

It has been shown in rats that ethanol consumption before and during pregnancy significantly decreases the number and size of offspring, by some mechanism apparently unrelated to calorie intake (*Cited in F.C.T.* 1976, **14**, 355).

The experiments described in the two papers cited above were performed on pregnant ewes. Infusion of 15 g ethanol/kg by intrajugular catheter as a 9·75% solution in 5% dextrose was performed for 1 or 2 hr, both maternal and foetal blood being sampled every 30 min during infusion and every 15 min thereafter. Maximum ethanol concentrations in maternal and foetal blood (2·3 and 2·2 g/litre, respectively) occurred at the end of the 1-hr infusion, both concentrations being 70% of their peak value 45 min later. When the infusion was given over 2 hr, ethanol concentrations reached maxima of 2·4 g/litre in the maternal blood at 90 min and 2·2 g/litre in the foetal blood at 120 min. One hour after infusion, both concentrations were still 75% of the maxima. The mothers showed hyperlactacidaemia and mild hyperglycaemia without significant acid–base disturbance, whereas, in the foetus, metabolic acidosis and a later progressive mixed acidosis appeared.

In parallel experiments, ethanol depressed the foetal blood pressure significantly during the 30-min period after the infusion. Cerebral blood flow in the foetus increased significantly as a result of decreased vascular resistance, while oxygen uptake was unaltered. Cerebral uptake of glucose and the glucose-oxygen utilization ratio increased. The amplitude and frequency of the dominant foetal electroencephalogram rhythm decreased with increasing concentration of ethanol in the blood; it did not recover after the infusion and became isoelectric (flat) as foetal acidosis increased. The authors suggest, therefore, that depressed central nervous activity should be anticipated in human neonates following unsuccessful attempts to inhibit premature labour by ethanol infusion.

PRESERVATIVES

3099. AF-2 suspicions confirmed

Sugiyama, T., Goto, K. & Uenaka, H. (1975). Acute cytogenetic effect of 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2, a food preservative) on rat bone marrow cells *in vivo*. *Mutation Res.* **31**, 241.

The nitrofurans as a class include a large number of demonstrable animal carcinogens. Consequently, the safety-in-use of the food preservative AF-2 (furylfuramide: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide), approved at one time for use in Japan, underwent very close scrutiny. Although at that time AF-2 gave no evidence of carcinogenicity in mouse and rat feeding studies (*Cited in F.C.T.* 1972, **10**, 717) and negative results have been reported recently in a dominant lethal study in mice (*ibid* 1976, **14**, 645), AF-2 did show a similar mutagenicity profile to other nitrofuran carcinogens when tested in bacterial systems (*ibid* 1976, **14**, 68). The study cited above investigated further the mutagenic potential of AF-2 *in vivo*.

The chromosomes of bone-marrow cells from the

femurs of male rats were studied after ip or oral administration of AF-2. Significant increases in the incidence of chromosomal aberrations were first apparent 4 hr after ip injection of 240 mg AF-2/kg, the incidence reaching a maximum after 6 hr and returning to control levels within 24 hr. A dose response was observed when chromosomal aberrations were scored 6 hr after ip administration of 4–240 mg AF-2/kg, a 9% incidence of aberrations being produced by the maximum dose compared with 1, 2 and 5% incidences in control animals and those given 15 and 120 mg AF-2/kg, respectively. Similar results were obtained when AF-2 was given by gavage, but 3–4 times the ip doses were necessary to elicit quantitatively similar degrees of chromosomal damage. The aberrations were shown to be basically similar to those induced by the carcinogen 7,12-dimethylbenz-[*a*]anthracene in terms of morphology and chromosomal distribution of breaks and gaps.

This experiment clearly showed the ability of AF-2 to damage DNA *in vivo*, and it is reported that the carcinogenicity of AF-2 has since been demonstrated by the production of stomach tumours in mice fed AF-2. A small increase in tumour incidence was also recorded in mice exposed to AF-2 during the foetal and neonatal stages (*ibid* 1976, **14**, 645). As a result of these findings, there has been a complete ban on the use of AF-2 in food in Japan since October 1974.

3100. Nitrosamines out of the frying pan

Sen, N. P., Donaldson, B., Seaman, S., Iyengar, J. R. & Miles, W. F. (1976). Inhibition of nitrosamine formation in fried bacon by propyl gallate and L-ascorbyl palmitate. *J. agric. Fd Chem.* **24**, 397.

The occurrence of highly carcinogenic nitrosamines in food products and their formation by the action of nitrosating agents on normal food and body constituents has been reviewed recently (Cooper, *Fd Cosmet. Toxicol.* 1976, **14**, 205). The inhibition by ascorbyl palmitate of the production of nitrosamines during the frying of bacon (*ibid* 1976, **14**, 167) has now been investigated further by the authors cited above.

Strips of bacon were sprayed with 5 ml of a solution containing about 1000 ppm of one of the following food additives: propyl gallate (PG), L-ascorbyl palmitate (AP), piperazine hydrate, sodium ascorbate, or nitrosoproline (NPro). After spraying, the samples were left for 30–60 min at room temperature before being fried to a 'well-done' stage. Control samples were not sprayed before cooking. Nitrosopyrrolidine (NPyr) was extracted with methylene chloride from the cooked bacon and cooked-out fat, analysed by a combination of thin-layer chromatography and fluorimetry, and confirmed by gas–liquid chromatography–mass spectrometry.

For most brands of bacon tested, PG, AP and piperazine hydrate inhibited the formation of NPyr in the cooked bacon by more than 70%, with sodium ascorbate inhibiting the formation to a lesser extent. The addition of cysteine or sodium ascorbate along with PG gave results similar to those obtained with PG alone. When lard or non-nitrite bacon (bacon prepared without nitrite) was cooked alone no NPyr was formed, but when either was cooked with NPro, levels of NPyr between 0·04 and 0·06 ppm were detected. AP, PG and piperazine hydrate had no observable effect on the yield of NPyr from NPro, which suggested that these additives inhibited NPyr formation in normal cooked bacon by interfering with reactions other than the decarboxylation of NPro. Small residual amounts of NPyr in the additive-treated bacon after cooking probably originated from NPro present before cooking.

The authors conclude that nitrosation reactions occurring during the cooking of bacon play a much greater part in the formation of NPyr than has been thought. As AP and PG are approved food additives, the addition of these to bacon before or after curing may be acceptable, but the feasibility of this treatment has not yet been determined.

PROCESSING AIDS

3101. Polydimethylsiloxanes in the environment

Hobbs, E. J., Keplinger, M. L. & Calandra, J. C. (1975). Toxicity of polydimethylsiloxanes in certain environmental systems. *Envir. Res.* **10**, 397.

Polydimethylsiloxanes (PDMSs) are effective antifoam agents used in the processing of certain foods such as bread and cake, in the manufacture of drugs and cosmetics and in a variety of other industrial applications. In earlier studies, members of this class showed no toxicity to the rabbit or rat (*Cited in F.C.T.* 1967, **5**, 263) and were not carcinogenic in the mouse (Cutler *et al. Fd Cosmet. Toxicol.* 1974, **12**, 443). The wide use of these compounds, however, and the possibility of their release into the environment, combined with their resistance to biodegradation, prompted the further study cited above.

Various Dow Corning PDMS fluids were studied for their toxicity, persistence and accumulation under different environmental conditions. In a simulated sewage digestor, [^{14}C]PDMS produced no $^{14}CO_2$ and, after 70 days, all the radioactivity was found on the filtered particulate matter, suggesting that no biodegradation or incorporation of PDMS by sewage bacteria had occurred.

The toxicity of the fluids to daphnia, fresh-water fish, marine species, mallard ducks, bobwhite quail and domestic chickens was very low. Residues in the fresh-water bluegill sunfish exposed to [^{14}C]PDMS in water for up to 31 days were low and highly variable (0·00–0·76 ppm in the tissue with exposures to 1 or 10 ppm) and showed no correlation with either duration or level of exposure. Since PDMS was shown to be deposited on the walls of the aquaria, the ^{14}C activity detected may have been due to surface contamination rather than to cellular uptake of the silicone.

In white Leghorn chickens fed PDMS at dietary levels up to 1000 ppm, neither egg production, quality

and hatchability nor the body weights and viability of the chicks were affected. Residues of silicon in the eggs and in the flesh of the chickens were below the limits of detection of 2–4 ppm.

The authors consider, therefore, that despite the resistance of these compounds to degradation and their consequent persistence in the environment, their low toxicity and apparent immobilization by binding to particulates in aqueous systems should minimize their potential for causing environmental damage.

AGRICULTURAL CHEMICALS

3102. Metabolism of ethylene thiourea

Ruddick, J. A., Williams, D. T., Hierlihy, L. & Khera, K. S. (1976). [^{14}C]Ethylenethiourea: Distribution, excretion, and metabolism in pregnant rats. *Teratology* **13**, 35.

Ethylene thiourea (ETU) is a contaminant and metabolite of the ethylenebisdithiocarbamate fungicides and has been shown to produce goitre and to be teratogenic in rats (*Cited in F.C.T.* 1973, **11**, 1144; *ibid* 1975, **13**, 584). The authors cited above report a study of the metabolism of a teratogenic dose of ETU in rats and discuss the implications of their results for the teratogenicity of ETU.

Female Wistar rats were given a single oral dose of 240 mg [^{14}C]ETU/kg on day 11, 12 or 15 of gestation. Rats dosed on days 11 or 12 were killed 6, 12 or 24 hr after treatment and those dosed on day 15 were killed after 3 hr.

The concentrations of ^{14}C in the maternal kidney and liver and in the embryos 6 and 12 hr after dosing on day 11 were 25, 23 and 12 dpm/mg, and at 24 hr the values were 3·7, 3·9 and 1·8 dpm/mg respectively. Maternal blood counts were 25,000, 21,000 and 22,000 dpm/ml at 6, 12 and 24 hr, respectively, after treatment. Results for treatment on day 12 were similar. Three hours after dosing on day 15, counts in maternal liver, kidney, muscle, placenta and embryo were similar to each other, with a mean of 81 dpm/mg. Counts in maternal blood and urine were 82,000 and 14,000 dpm/mg, respectively. On day 15 the half-life of ^{14}C in maternal blood was calculated to be 10 hr. The ^{14}C label was distributed evenly between the red blood cells and the plasma. Of the total ^{14}C given on day 15, 55% was excreted by 12 hr, 73% by 24 hr and 84% by 48 hr after treatment, mostly in the form of [^{14}C]ETU.

The authors conclude from the lack of degradation that ETU is a teratogen *per se*.

3103. Filling in the HCB picture

Koss, G. & Koransky, W. (1975). Studies on the toxicology of hexachlorobenzene. I. Pharmacokinetics. *Arch. Tox.* **34**, 203.

Mollenhauer, H. H., Johnson, J. H., Younger, R. L. & Clark, D. E. (1975). Ultrastructural changes in liver of the rat fed hexachlorobenzene. *Am. J. vet. Res.* **36**, 1777.

Since our last review of hexachlorobenzene (HCB) was published (Cooper, *Fd Cosmet. Toxicol.* 1976, **14**, 351), two more reports on its metabolism and toxicity have reached us.

The first paper cited emphasizes the influence of the vehicle on the body distribution of HCB. Female rats were given [^{14}C]HCB orally as a 0·2–1·2% solution in olive oil or as a 0·4–20% aqueous suspension. Absorption of HCB from olive oil accounted for 80%, irrespective of the dose, which varied from 20 to 180 mg HCB/kg. From aqueous suspension, absorption was 20, 5 and 2% with doses of 16, 120 and 970 mg/kg, respectively. All the tissues of animals given HCB in olive oil contained ^{14}C, the activity of which reached a maximum after 2–5 days; the highest activity was in adipose tissue and the lowest in the muscle and blood. When rats were given a single ip injection of 4 mg [^{14}C]HCB/kg in olive oil, 34% of the dose was excreted in the faeces and 5% in the urine within 14 days, about 80 and 4%, respectively, being unchanged HCB. No radioactivity was detected in the expired air.

The second paper cited describes the effects on the ultrastructure of the liver of feeding 1–25 ppm HCB to rats for up to 12 months. No gross liver changes appeared, but after 5, 10 or 25 ppm HCB had been fed for 3 months or longer, ultrastructural changes were observed and these were related in magnitude to the total dose. In some cases, usually in conjunction with the lower doses, smooth endoplasmic reticulum proliferated, but in other cases there was an increased deposition of glycogen. Elongation or swelling was observed in some mitochondria, and storage bodies, possibly remains of degenerated mitochondria, developed in some cells. No such changes followed feeding with 1 ppm HCB.

3104. Naphthol in the river

Bollag, J.-M., Czaplicki, E. J. & Minard, R. D. (1975). Bacterial metabolism of 1-naphthol. *J. agric. Fd Chem.* **23**, 85.

1-Naphthol is a major hydroxylation product of carbaryl, and appears in the urine of persons exposed to that insecticide (*Cited in F.C.T.* 1976, **14**, 214). Since it may be more toxic than carbaryl to some organisms in the ecosystem, its fate when exposed to soil and water micro-organisms is of considerable importance.

In the study cited above, two micro-organisms which were active in degrading 1-naphthol were isolated from river water. They were not fully characterized or identified but were shown to be gram-negative aerobic rods, one non-flagellate and the other possessing between one and four flagella. Neither organism fermented glucose but both caused an alkaline shift

when grown on sucrose, lactose or maltose. They were incubated with 20, 50 or 100 ppm [1-^{14}C]naphthol, since higher concentrations of naphthol completely inhibited their growth. After incubation for 60 hr, 44% of the radioactivity was present in evolved carbon dioxide, 17% remained in the medium and 22% was in the bacteria themselves. Extraction of the culture medium with diethyl ether, followed by thin-layer chromatography of the extract showed the major bacterial metabolite to be 4-hydroxy-3,4-dihydro-(2-*H*)-naphthalenone.

The production of carbon dioxide shows that micro-organisms can break the naphthyl ring of 1-naphthol, but the persistence of 15–20% of the radioactivity in the medium in the form of metabolites which did not undergo further change indicates that there must be at least one other metabolic pathway by which naphthol can be degraded by water-borne bacteria.

PROCESSING AND PACKAGING CONTAMINANTS

3105. Re-examination of the dioctyl tins?

Seinen, W. & Willems, M. I. (1976). Toxicity of organotin compounds. I. Atrophy of thymus and thymus-dependent lymphoid tissue in rats fed di-n-octyltindichloride. *Toxic. appl. Pharmac.* **35**, 63.

The alkyltins are used widely in industry as stabilizers, catalysts and biocides. Although several dioctyltin compounds are permitted in the USA as additives in PVC destined for food contact, the dioctyltins as a class have not undergone extensive toxicological examination. Di-*n*-octyltin dichloride (DOTC) exhibited no adverse effects when fed to rats at a dietary level of 200 ppm for 4 months (Barnes & Stoner, *Br. J. ind. Med.* 1958, **15**, 15), but in a study previously reviewed (*Cited in F.C.T.* 1974, **12**, 574), similar dietary levels of di-*n*-octyltin *S,S'*-bis(isooctyl mercaptoacetate) given to rats over 12 months produced increased kidney weights and decreased body weights.

In the study cited above, rats were fed diets containing 50 or 150 ppm DOTC for 6 wk. Rats on the higher treatment level showed evidence of renal impairment, exemplified by a significant increase in the blood urea-nitrogen level of the males and a decrease in the specific gravity of the urine of the females. At the higher dietary level, the animals also exhibited a significant decrease in food efficiency, and the males had a low haemoglobin level. There was a dose-related increase in serum alkaline-phosphatase activity in all treated animals, and a decrease in the activities of serum glutamic–pyruvic and glutamic–oxalacetic transaminases in the females of both treated groups.

The main pathological findings concerned the thymus and the thymus-dependent areas in the peripheral lymphoid organs. Thymic atrophy occurred in both the treated groups, the reduction in organ size being more pronounced in the animals fed 150 ppm DOTC, at which level the thymus was almost completely devoid of lymphocytes. Similar but less severe depletion occurred with 50 ppm DOTC. Total counts of thymus cells and cell viability data, obtained from a supplementary 4-wk feeding study, indicated that DOTC treatment produced an early inhibition of cell division followed by cell death.

There were no histological signs of hyperactivity in the adrenal gland of the treated animals, and the relative adrenal weights were similar in both the DOTC-fed and the control animals. In a further study using adrenalectomized male rats, DOTC in the diet at 150 ppm again decreased thymus weights. The possibility of stress-initiated changes in the thymus was therefore discounted.

The authors consider this to be the first report of the highly selective toxicity of these alkyltin compounds to the thymus, and, consequently, further immunological studies on this phenomenon are being undertaken.

MISCELLANEOUS CONTAMINANTS

3106. Mercury and cell growth

Nakazawa N., Makino, F. & Okada, S. (1975). Acute effects of mercuric compounds on cultured mammalian cells. *Biochem. Pharmac.* **24**, 489.

The problems of mercury as an environmental pollutant and, more specifically, as a potential food contaminant, need no introduction to readers of this journal. Considerable attention has been paid to the importance of alkylmercury compounds in this connexion, and to the conditions under which the alkylation of inorganic mercury compounds is most likely to occur (*Cited in F.C.T.* 1974, **12**, 581). The overt and subclinical effects of both organic and inorganic mercury compounds in man and experimental animals have been reported widely, but less is known about the effects of mercury at the cellular level, although some years ago lymphocyte cultures from a group of men with high tissue levels of mercury were found to show a high incidence of chromosomal aberrations (Skerfving *et al. Archs envir. Hlth* 1970, **21**, 133).

In the study by Nakazawa *et al.* (cited above), mouse leukaemia L5178Y cells were cultured to the exponential-growth phase and then exposed for 60 min to mercuric chloride ($HgCl_2$), methylmercury chloride (MeHgCl) or phenylmercury acetate with and without [^{3}H]thymidine or [^{3}H]uridine, and the subsequent growth was observed.

Cell growth was not affected by 10^{-6} M-MeHgCl or 10^{-5} M-$HgCl_2$ but at 4×10^{-6} and $2{\cdot}5 \times 10^{-5}$ M, respectively, these compounds completely prevented

growth during the 3-day observation period. Incorporation of [^{3}H]thymidine into the acid-insoluble cell fraction was depressed by 2×10^{-7} M-MeHgCl, 2×10^{-7} M-phenylmercury acetate and 2×10^{-6} M-$HgCl_2$. All three compounds similarly depressed incorporation of [^{3}H]uridine. Glutathione (0·5 mM) added to the culture medium reduced the degree of retardation of cell growth and increased cell survival in the presence of MeHgCl. The concentration of these mercury compounds required to induce single-strand scissions of DNA was about ten times greater than that required to depress DNA synthesis.

THE CHEMICAL ENVIRONMENT

3107. Aluminium toxicity in renal failure

Alfrey, A. C., LeGendre, G. R. & Kaehny, W. D. (1976). The dialysis encephalopathy syndrome. Possible aluminum intoxication. *New Engl. J. Med.* **294,** 184.

Elevated serum levels of aluminium (Al) have been found in uraemic patients undergoing dialysis and receiving oral Al to control serum-phosphorus concentrations, and this finding has also been demonstrated in nephrectomized rats given Al hydroxide by gavage or Al salts in their drinking-water (*Cited in F.C.T.* 1973, **11,** 140). The latter treatment produced various toxic effects, including periorbital bleeding. Other uraemic patients receiving Al showed a positive Al balance of 100–568 mg/day during treatment, although their serum-Al levels were not consistently affected and slightly elevated bone levels of Al were found in only two of these patients (Clarkson *et al. Clin. Sci.* 1972, **43,** 519). In another study, elevated bone levels of Al were demonstrated in ten patients undergoing chronic dialysis and six of these were found to be suffering from polyneuropathy (Parsons *et al. Br. med. J.* 1971, **4,** 273). Further evidence that Al may accumulate in the body and damage the central nervous system after prolonged administration to uraemic subjects has now come to light.

An outbreak of encephalopathy in the Denver area among uraemic patients who had been maintained on chronic dialysis and given about 2 g Al as the hydroxide daily for at least 2 yr, prompted an investigation of Al levels in the tissues of these subjects (group 1), of dialysed uraemic patients not suffering from encephalopathy (group 2) and of normal subjects (group 3). In the brain the mean Al level in the grey matter was as high as 24·98 ppm in six group 1 subjects, compared with only 6·5 ppm in seven subjects of group 2 and 2·18 ppm in five of group 3. The white matter showed a similar but far less marked trend, mean Al levels of 5·59, 3·81 and 2·00 ppm, respectively, being found in the three groups. Muscle levels resembled those in the grey matter, with mean levels of 23·60 ppm in ten patients of group 1, 10·24 ppm in 14 of group 2 and 1·22 ppm in 13 of group 3. Not enough bone samples were obtained from encephalopathic patients to permit separate analysis but in 16 subjects from groups 1 and 2 combined the mean Al levels were as high as 98·48 ppm in trabecular bone and 46·83 ppm in cortical bone, far above the mean levels of 2·39 ppm and 3·88 ppm, respectively, in nine normal subjects. In three uraemic patients not undergoing dialysis, intermediate levels of 37·4 ppm and 8·4 ppm, respectively, were detected. In addition there was a significant correlation between the duration of dialysis and the Al content of bone, muscle and grey matter but not of white matter. The Al content of the dialysate itself was negligible, suggesting that the tissue residues were derived from Al taken orally.

Encephalopathy was first noted in Denver patients undergoing dialysis in 1971, about 2·5 yr after the start of routine prescription of Al hydroxide for controlling serum phosphorus, and 14 cases have since occurred. The disease has been described in patients from several different units, and in some has been the commonest cause of death. Most of these patients had been on intermittent dialysis for 3–7 yr before the onset of symptoms, and the clinical features, which were similar in all patients, suggested involvement of the grey matter in which Al has now been shown to accumulate. Encephalopathy has occurred in an industrial worker exposed to Al (McLaughlin *et al. J. ind. Med.* 1962, **19,** 253) and toxic effects of Al on nervous tissue *in vitro* or after direct application to the brain *in vivo* have been described elsewhere. The authors of the present paper suggest that serum phosphorus in uraemic patients undergoing chronic dialysis should be controlled by means other than Al administration, and propose magnesium-containing antacids as a possible alternative.

3108. The nickel-plated lung

Wehner, A. P., Busch, R. H., Olson, R. J. & Craig, D. K. (1975). Chronic inhalation of nickel oxide and cigarette smoke by hamsters. *Am. ind. Hyg. Ass. J.* **36,** 801.

Apart from nickel dermatitis, most manifestations of nickel intoxication result from the inhalation of aerosols of the metal or its compounds. Nickel oxide (NiO) has a relatively low toxicity; inhalation of 184 μg/litre produced no toxic effects in hamsters, although the animals showed considerable lung deposits of NiO 1 wk after exposure (*Cited in F.C.T.* 1973, **11,** 1145). The likelihood of increasing human exposure to nickel in urban atmospheres has prompted the authors cited above to investigate the possible role of NiO, in conjunction with tobacco smoke, in producing pneumoconiosis and lung cancer.

Syrian golden hamsters were exposed to a respirable aerosol of NiO for 7 hr/day, 5 days/wk for their life-span. Some were exposed additionally to cigarette smoke for 20 min before and 10 min after each exposure to NiO. The first effect observed was an accumulation of NiO particles on the alveolar septa, the location of the particles being both extra- and intracellular. The NiO accumulated initially in macrophages

adjacent to small blood vessels, lymph vessels and small bronchioles. From animals dying early in the experiment, it was apparent that emphysema developed before the onset of pneumoconiosis. Later deaths were associated with the appearance of cellular proliferation and inflammation of the lungs, with increasing degrees of consolidation. Exposure to cigarette smoke in addition to NiO made no marked difference to the pathological picture, except for the appearance of macrophages with brownish cytoplasmic inclusions ('smoke cells') in the lungs and an increase in inflammatory lesions of the larynx. There was no evidence of any carcinogenic effect of NiO dust, nor of any co-carcinogenic effect of tobacco smoke. However, the exposure to cigarette smoke with or without exposure to NiO significantly lowered the body weight of the animals, and appeared to prolong their survival—a curious observation which the authors claim has not previously been reported.

3109. Nickel and the epidermis

Samitz, M. H. & Katz, S. A. (1976). Nickel–epidermal interactions: Diffusion and binding. *Envir. Res.* **11**, 34.

Several suggestions have been made to explain the mode of action of nickel in contact sensitivity (*Cited in F.C.T.* 1976, **14**, 508), but few experimental data on the interactions of nickel with skin and their possible relationship to nickel sensitivity have been reported. In the paper cited above, both the diffusion of nickel through the epidermis under various conditions and the effects of chemical modification of the epidermis on the binding of nickel were investigated in order to elucidate the part played by nickel–epidermal interactions in hypersensitivity responses.

An *in vitro* study using radioactive nickel (^{63}Ni) examined the diffusion of labelled nickel sulphate solutions at concentrations of 0·1, 0·01 and 0·001 M in physiological saline and 0·001 M in sweat or 2% surfactant solution. Results indicated that no diffusion of nickel through the epidermis from physiological saline took place within 5 hr. Sweat or surfactant enhanced the diffusion of the nickel only slightly; after a period of 48 hr, less than 0·1% of the nickel had diffused through the epidermis.

In the binding experiments, epidermis powder was prepared from autopsy skin. One portion was retained as control epidermis and the others were modified by chemical means to block carboxyl groups by methylation or benzoylation, to inactivate amino groups by acetylation or deamination or to block sulphydryl groups. The epidermal powder samples were added to solutions of labelled nickel sulphate and incubated at 0°C for 2 wk, after which the powder and solution were separated and the powder was washed with saline. The decrease in activity of the solution compared with that of solutions with no added epidermis powder and the increase in activity of the washed powder was used to give separate estimations of the amount of nickel bound to the epidermis.

Although the results of these two methods of estimation did not show quantitative agreement, estimates based directly on the activity of the washed powder being consistently lower, it appeared that the nickel was bound primarily by free carboxyl groups. The authors suggest that the binding was weak and reversible, and that this could explain the differences observed in the two estimations, since the washing procedure could have resulted in the removal of weakly bound nickel. Results also indicated the involvement of amino and sulphydryl groups in nickel binding, although this was less marked than the influence of carboxyl groups.

3110. Myocardial effects of acrylic bone cements

Yasuda, I. & Iwatsuki, K. (1975). Direct effects of acryl bone cement monomer on isolated heart muscle. *Tohoku J. exp. Med.* **117**, 93.

The use of polymethyl methacrylate in the fixation of total joint replacements has been a major development in orthopaedic surgery, but there have been reports of severe cardiovascular reactions to this surgical procedure. Numerous suggestions have been put forward in an attempt to identify the factors involved in the development of these reactions, but a recent review on the subject of acrylic cement reactions (Cooper, *Fd Cosmet. Toxical.* 1975, **13**, 390) underlined the uncertainties that remain in this field.

Prominent among the points at issue has been the suggestion that absorption of free monomer from the acrylic bone cement into the systemic circulation might give rise to these cardiovascular complications. The study cited above investigated the direct effects of the liquid acrylic monomer used to produce bone cement *in situ* on the contractility of isolated canine heart muscle. Concentrations of 0·03, 0·06 and 0·12 ml/100 ml were added to the muscle-bathing solution and the velocity of muscle shortening and the force developed in the myocardium were measured. A concentration of 0·03 ml/100 ml corresponds roughly to the blood concentration when 0·5 ml of the monomer is injected into a 20-kg dog with an assumed blood volume of 70 ml/kg.

Results indicated a leftward shift in the force–velocity relation curves, a decrease in the maximum force developed and a concomitant dose-dependent decrease in cardiac work and power, the depression in work being the greater. These findings imply that the monomer produces a direct negative inotropic effect on the heart muscle. This decrease in the contractility of the myocardium contrasts with the observations in some earlier studies (Peebles *et al. Br. med. J.* 1972, **1**, 349) in which a significant fall in arterial blood pressure accompanied by an increase in heart rate was tentatively attributed to peripheral vasodilation caused by the monomer. It is known that the cardiovascular depression caused by the monomer is transient and may be counteracted by sympathetic activity, but in patients with some impairment of compensatory mechanisms, there is likely to be a relatively high risk of cardiovascular complication following surgery involving acrylic bone cement.

3111. Solvents, salves and sensitization

Pevny, I. & Uhlich, M. (1975). Allergie gegen Bestandteile medizinischer und kosmetischer Externa: Polyäthylenglykol, Propylenglykol, Stearylalkohol, Hexantriol. *Hautarzt* **26,** 252.

Both stearyl alcohol and propylene glycol have been found to be capable of producing primary irritation and dermatitis (*Cited in F.C.T.* 1975, **13,** 403). This ability is confirmed in the report cited above.

Eight of the cases of contact dermatitis seen in a dermatological clinic during a period of 1·5 yr were attributed to the application of a skin ointment (Topsym), and this was confirmed by patch-testing with the preparation. When the components of the ointment were tested separately on these patients, positive reactions to stearyl alcohol (20% in vaseline) were recorded in five of the eight and the incidence of positive reactions to propylene glycol (10% in water) was the same, although the individuals concerned were not the same in every case. Three members of this group of eight gave positive reactions to hexanetriol (5% in water) but none of these patients reacted to polyethylene glycol 1500. One of the two patients who gave positive reactions to stearyl alcohol, propylene glycol and hexanetriol also showed a general sensitization to low-molecular-weight alcohols.

The authors claim that cases of sensitivity to hexanetriol have not previously been reported.

3112. TLV for formaldehyde questioned

Schoenberg, J. B. & Mitchell, C. A. (1975). Airway disease caused by phenolic (phenol–formaldehyde) resin exposure. *Archs envir. Hlth* **30,** 574.

Phenol–formaldehyde (PF) resins as a class have one of the longest careers in the polymer industry. Reports of the toxicity of these materials have been noticeably few, although a recent study did implicate bakelite dust (a PF resin) as the cause of pulmonary fibrosis in a number of subjects (*Cited in F.C.T.* 1975, **13,** 479). While it seems that the polymer itself is relatively inert biologically, the comonomers phenol and formaldehyde are much more hazardous materials.

The study cited above reports on the adverse effects of "phenol–formaldehyde resin fumes" in a number of workers involved in operating a process consisting of dipping acrylic-wool fibres into liquid PF resin and curing the resultant product in an oven at 160°C. A typical worker is exposed to this process on alternate working shifts. A group of 40 process workers was compared with eight employees who were not process workers at that time but who had been in the past and with 15 control employees who had never been routinely involved with the actual PF process. Smoking habits, respiratory history and the workers' performance in a number of tests of pulmonary function were assessed in relation to occupational exposure. Although there had been no systematic measurement of monomer levels over the years, typical plant levels were thought to be 0·5–1 mg/ml^3 for formaldehyde and 7–10 mg/m^3 for phenol. In abnormal situations, such as an extractor-fan breakdown, formaldehyde levels of 10–16 mg/m^3 (4–6 times the threshold limit value) had been recorded.

The current process workers showed a higher incidence of coughing and phlegm production than the other groups, and the majority of these workers also complained of excessive lachrymation and conjunctival, nasal and throat irritation as a result of fume exposure. Other lower-respiratory-tract symptoms in addition to cough were reported by many process workers, but by only a single control subject. Respiratory symptoms were said to occur typically within minutes of a brief heavy exposure and to decrease some 5–15 min later. Delayed onset of symptoms did occur, however, in seven subjects, and this was accompanied by a prolonged duration of discomfort. Current workers who had been exposed to the process for longer than five continuous years exhibited a statistically significant decrease in performance in the pulmonary tests compared with the performance of control workers ($P < 0{\cdot}005$ when the data had been normalized for cigarette consumption).

In spite of the limitations of the study, of which the investigators were well aware, the results suggested that long-term exposure to "phenol–formaldehyde resin fumes" produced chronic airway obstruction. As no changes in respiratory performance could be detected after a shift or working week, a further study is planned in the same subjects to ascertain whether measurable deterioration of health is occurring with time. In the study already completed, there were no apparent differences in the data from control subjects and from those workers formerly but no longer involved with the process, indicating that the chronic effects of the fumes may be reversible.

[Although the title of the paper indicts phenol–formaldehyde resin as the cause of the airway disease observed in these workers, both the process description and the reported toxic effects indicate that formaldehyde toxicity was the factor in question. Accurate assessment of exposure levels was not possible, but it seems that the airway disease was occurring with permissible atmospheric concentrations of formaldehyde.]

3113. TDI and the lungs

VanErt, M. & Battigelli, M. C. (1975). Mechanism of respiratory injury by TDI (toluene diisocyanate). *Ann. Allergy* **35,** 142.

Toluene diisocyanate (TDI) is known to cause bronchial reactions in workers exposed to it in plants manufacturing polyurethane foam (*Cited in F.C.T.* 1972, **10,** 730). Although sensitization has been considered as a step in this reaction, the mechanism of TDI-induced bronchial disease is still obscure. In the paper cited above, evidence is presented that TDI does not release histamine directly but may contribute to the action of histamine and other mediators by moderating β-adrenergic function.

Peripheral leucocytes from atopic or non-atopic individuals failed to release histamine when treated with TDI, and TDI in concentrations of more than 10^{-5} M inhibited the antigenic release of histamine from such cells. Lower concentrations of TDI (10^{-7}–10^{-5} M)

reinforced the inhibitory effect of isoprenaline on the antigenic release of histamine but higher concentrations did not. Concentrations of 50–100 mM-TDI inhibited the increase in cyclic-AMP induced by the action of the catecholamines, isoprenaline, adrenaline or noradrenaline, on the leucocytes. Thus, there is no evidence that TDI *per se* possesses histamine-releasing activity, but it appears to moderate the β-adrenergic function. It resembles propranolol in reducing cyclic-AMP induction by catecholamines, but differs from it in inhibiting cyclic-AMP induction by glucagon and in inhibiting the antigenic release of histamine.

3114. Triaryl phosphate poisoning in fish

Lockhart, W. L., Wagemann, R., Clayton, J. W., Graham, B. & Murray, D. (1975). Chronic toxicity of a synthetic tri-aryl phosphate oil to fish. *Envir. Physiol. Biochem.* **5**, 361.

The toxicity and metabolism of the triaryl phosphates have been well documented (*Cited in F.C.T.* 1969, **7**, 701; *ibid* 1975, **13**, 676) and electron microscopy has indicated the sequence of pathological changes that occur as a result of poisoning. However, the biochemical aspects of the toxicology of these compounds have not been fully elaborated. The authors cited above have attempted to clarify the biochemical and other effects that follow the exposure of fish to IMOL S-140, a synthetic high-temperature lubricating oil composed of triaryl phosphates.

Rainbow trout were exposed to 0·9 ppm IMOL S-140 in the water flowing to them over a period of 4 months. During this time, the fish were observed for tissue accumulation of IMOL S-140, blood-serum abnormalities, brain-cholinesterase activity and behavioural modifications. In an assay of acute toxicity, guppies (7–9 days old) were exposed for 24 hr in standing water saturated with IMOL S-140.

No guppies were killed in the acute toxicity test. Treated rainbow trout failed to consume normal rations of floating pellets after 8 days, but in spite of this, growth in both length and weight continued and feeding may have continued unobserved on sunken food pellets. The activities of glutamic–oxalacetic transaminase and lactate dehydrogenase in serum were greatly elevated, and internal fatty tissues became discoloured. Neither serum- nor brain-cholinesterase activities were inhibited.

Cholinesterase activity, particularly in brain tissue but also in serum, has been considered the most appropriate test for detecting poisoning of fish by organophosphorus pesticides. The diagnostic value of cholinesterase activities in cases of triaryl phosphate poisoning in birds and mammals has been questioned (Bondy *et al. Toxicology* 1973, **1**, 143) and recently the pesticide phosvel has been found to exhibit the delayed toxicity typical of tri-*o*-cresyl phosphate (Abou-Donia *et al. Experientia* 1974, **30**, 63). The present results have shown that IMOL S-140 or at least one of its components has toxic properties not likely to be discovered by standard 96-hr bioassay procedures nor, indeed, by the serum- and brain-cholinesterase measurements often used in tests of poisoning by organophosphates.

3115. Mutagenicity of chlorinated ethylenes

Greim, H., Bonse, G., Radwan, Z., Reichert, D. & Henschler, D. (1975). Mutagenicity *in vitro* and potential carcinogenicity of chlorinated ethylenes as a function of metabolic oxirane formation. *Biochem. Pharmac.* **24**, 2013.

It has been suggested that the active carcinogenic metabolite of vinyl chloride is the epoxide, chloroethylene oxide (*Cited in F.C.T.* 1976, **14**, 499). A similar compound has been suggested as an important intermediate in the metabolism of trichloroethylene, recently implicated as a carcinogen in mice (Seltzer, *Chem. Engng News* 1975, **53** (20), 41). Chloroethylene oxide and chloroacetaldehyde, a further metabolic product of vinyl chloride, are both strongly mutagenic in Salmonella (*Cited in F.C.T.* 1976, **14**, 500), as is a metabolic product of the related compound, vinylidene chloride (*ibid* 1976, **14**, 220). The mutagenicity of all the chlorinated ethylenes before and after metabolic activation has now been assessed.

Tetrachloroethylene, trichloroethylene, 1,1-dichloroethylene (vinylidene chloride), *cis-* and *trans*-1,2-dichloroethylene and vinyl chloride were incubated for 2 hr at 37°C with *Escherichia coli* K 12, alone or with hepatic microsomal protein obtained from mice (pretreated for 10 days with phenobarbitone to increase microsomal-enzyme activity) and an NADPH-generating system. Concentrations of the chlorinated ethylenes were such that they did not reduce bacterial survival by more than 25%, and were in the range 2·3–3·3 mM apart from tetrachloroethylene (0.9 mM) and vinyl chloride (10·6 mM). Mutagenicity was assessed, after plating the bacteria, in terms of increases in the rate of three back mutations (gal^+, arg^+ and nad^+) and a forward mutation involving resistance to 5-methyl-DL-tryptophan.

None of the compounds showed mutagenic activity without metabolic activation. However, after metabolic activation vinyl chloride produced large increases in all four types of mutation, and trichloroethylene and vinylidene chloride produced lesser increases in three and two, respectively, of the back mutations investigated. None of the remaining three compounds gave evidence of metabolism to mutagenic metabolites. These last three have been shown by other workers to give rise to fairly stable epoxides, whereas the epoxides formed from vinyl chloride, 1,1-dichloroethylene and trichloroethylene are structurally unstable, and it is suggested that this difference may be responsible for the differences in mutagenic activity.

[As indicated above, an epoxide is not the only mutagenic metabolite of vinyl chloride. Whether it is the epoxide itself or a decomposition product that is the mutagenic derivative of the chlorinated ethylenes would need to be verified by studies on each of the metabolites.]

3116. Behavioural effects of trichloroethylene

Silverman, A. P. & Williams, H. (1975). Behaviour of rats exposed to trichloroethylene vapour. *Br. J. ind. Med.* **32**, 308.

Humans exposed to trichloroethylene (TCE) at a concentration of 200 ppm complained of fatigue,

sleepiness and the need for greater mental effort in performing a balancing test, but the actual results of all performance and neurological tests conducted were normal (*Cited in F.C.T.* 1971, **9,** 452). However, in another study involving exposure to 110 ppm, a level only slightly above the present TLV of 100 ppm, there was a significant decline in performance, particularly in the more complex tests (*ibid* 1972, **10,** 116). In rats, prolonged exposure to a concentration of 400 ppm not only reduced performance in a swimming test, indicating behavioural depression, but also stimulated exploratory behaviour in a maze, a finding possibly attributable to a reduction in anxiety (*ibid* 1964, **2,** 225).

In the present study, pairs of male rats were exposed to TCE for 6–7 hr/day, 5 days/wk, for about 5 wk at concentrations in the range 100–1000 ppm and for 12·5 wk at a concentration of 100 ppm. Social behaviour of each pair during the first 5 min following each exposure period was assessed after treatment for 1, 3 and 7 days, and thereafter at weekly intervals. In all groups there was a consistent decline of up to 24% in total activity, which was evident after only 1 day at 1000 ppm but did not become significant until after 10 days in one experiment at 100 ppm and 59 days in the other. The reduction in activity was general and was usually not due to disproportionate reductions in specific kinds of behaviour, although cage exploration and submission to and/or escape from the other rat were sometimes specifically reduced. On the other hand, TCE-exposed rats that were deprived of water overnight and placed in an unfamiliar cage tended to find the water and begin drinking sooner than unexposed controls, although their rate of movement round the cage was unaffected.

Taken in conjunction with the findings of other workers, these results suggest that TCE reduces the performance of rats in a familiar situation but causes some loss of inhibitory control in an unfamiliar one. It would seem desirable, therefore, to investigate whether humans exposed to TCE concentrations around the TLV of 100 ppm tend to perform more slowly or less accurately in routine tasks, and whether they are more likely to make a hasty response in an unfamiliar (or perhaps emergency) situation.

3117. Spray adhesives exonerated again

Murphy, J. C., Collins, T. F. X., Black, T. N. and Osterberg, R. E. (1975). Evaluation of the teratogenic potential of a spray adhesive in hamsters. *Teratology* **11,** 243.

The claim, in 1973, that an increased rate of chromosome breakage occurred in persons exposed to spray adhesives could not be substantiated, and the temporary ban on sales of the adhesive in the USA was withdrawn (*Cited in F.C.T.* 1975, **13,** 156 & 405; *Federal Register* 1974, **39,** 3582).

The paper cited above describes the investigation of a spray adhesive containing 14% butadiene–styrene rubber and a resin derivative in a petroleum hydrocarbon solvent. Groups of 20 pregnant Syrian golden hamsters were exposed in an inhalation chamber to a total of 8·0, 11·6 or 13·2 g of the spray for two periods of 30 min on each of days 5–10 of gestation. The animals were killed and examined on day 14. The exposed animals were quiet and less active and aggressive than controls. Moreover, all those exposed to 13·2 g of spray and 17 of those exposed to 11·6 g developed alopecia within 4 days, and one of the former group died. In spite of the material's obvious toxicity to the pregnant animals, however, the mean numbers of foetuses and resorptions/litter did not differ from the control values. At all dose levels, the mean foetal weight was decreased and foetal development was retarded, but the delay in skeletal development was not dose related. No malformations attributable to exposure to the spray adhesive were observed.

3118. Synthetic fibres in the air

Pimentel, J. C., Avila, R. & Lourenço, A. G. (1975). Respiratory disease caused by synthetic fibres: a new occupational disease. *Thorax* **30,** 204.

There has been controversy over the respirability of fibres encountered in industry, but the indications are that any fibre less than 3·5 μm in diameter and 50 μm in length is capable of reaching the lungs (*Cited in F.C.T.* 1976, **14,** 361).

The study cited above describes seven cases of respiratory disease which occurred among textile workers handling synthetic fibres or making clothes from synthetic fabrics. Microscopy of lung tissue did not show the cause of the disease, which was characterized by an asthmatic syndrome, interstitial lung changes resembling those of allergic alveolitis, chronic bronchitis and bronchiectasis and spontaneous pneumothorax, and sometimes by X-ray shadows indistinguishable from those of lung cancer. Histochemical methods were applied to biopsy or autopsy specimens, and the characteristics of inclusions were compared by polarized-light microscopy with those of fresh fibres and paraffin sections. Specific precipitins were not detected, and skin and nasal provocative tests were negative for antigens derived from the fabrics. The prognosis was good in patients with the early, acute form of lung disease but was poor wherever widespread irreversible fibrosis appeared. The initiation of pulmonary disease from inhaled fabric particles seemed to be a highly individual reaction, which may have had an immunological basis.

When guinea-pigs were exposed to high concentrations of the dust of nylon or acrylic fibres for up to 325 days, 14 of 28 exposed to nylon and all ten exposed to acrylic dust developed lung lesions localized in the interalveolar septa. These comprised oedema, reticular fibrosis and granulomatous cellular proliferation of histiocytes and fibroblasts. There was no appreciable difference in the lung lesions associated with nylon or acrylic dust.

BIOCHEMICAL PHARMACOLOGY

3119. Comparative metabolism of drugs

Litterst, C. L., Mimnaugh, E. G., Reagan, R. L. & Gram, T. E. (1975). Comparison of *in vitro* drug metabolism by lung, liver, and kidney of several common laboratory species. *Drug Metab. Dispos.* **3,** 259.

Species differences in metabolic pathways for drugs make it difficult to extrapolate results from one species to another (*Cited in F.C.T.* 1976, **14,** 222). A study intended to simplify these extrapolations has now been undertaken using enzyme preparations isolated from the lung, liver and kidney of various species.

When microsomal and soluble (105,000-**g** supernatant) fractions from the organs of rats, hamsters, mice, rabbits and guinea-pigs were incubated with a range of drugs, the liver was shown to be the most active organ in all species, lung and kidney activities being some 15–40% of that of the liver. No consistent correlation could be established between the cytochrome *P*-450 level in the liver and the ability of different species to oxidize drugs, but in hamsters the relatively high NADPH-cytochrome *c* reductase activity correlated with generally high levels of drug oxidation. Levels of activity of *N*-acetyltransferase and of aniline and biphenyl hydroxylases were very low in lung tissues, except in rabbits in which biphenyl-hydroxylase activity was relatively high. Mouse lung was relatively rich in glutathione *S*-aryltransferase. The enzyme systems of the kidney, although more active than those of the lung, were much less active than those of the liver. No significant ultrastructural differences could be seen in the hepatic microsomes from different animal species and the same was largely true of the other microsomal fractions. Lung microsomes from all species showed aggregates of vesicles in a microfibrillar matrix, and there was a tendency towards similar aggregation in the kidney microsomes.

[In view of the wide range of enzymes and reactions involved, it is not particularly surprising that no single species appeared to be consistently superior for hepatic or extrahepatic drug-metabolism studies. It is of interest, however, that the rat—a species widely used in metabolic research and other aspects of toxicology—appears to be relatively deficient in overall drug-metabolizing activity in comparison with the other species studied.]

TOXICOLOGY

3120. Absorption of macromolecules in the newborn

Martinsson, K. & Jönsson, L. (1975). On the mechanism of intestinal absorption of macromolecules in piglets studied with dextran blue. *Zentbl. VetMed. A* **22,** 276.

Newborn animals have a marked capacity for intestinal absorption of macromolecules, but this facility is normally greatly reduced within a few days of birth (Morris, in *Handbook of Physiology*, Sec. 6, vol. III, edited by C.F. Code, p. 1491, American Physiological Society, Washington, 1968). The mechanism of absorption has now been studied in piglets, using dextran blue (mean mol wt 2×10^6) as a model compound.

Dextran blue was administered by gavage to piglets at birth (before suckling) or 6, 12, 24, 72 or 144 hr after birth. Examination at intervals up to 24 hr after administration revealed that in newborn animals the dye entered the mucosa, mostly in the lower half of the intestine, and was present in the regional lymph nodes within 2–3 hr. Uptake by the lymph nodes was still evident in 6- and 12-hr-old animals, and in these and in newborn piglets the dye was present also in the liver, spleen and body lymph nodes and, in some cases, in the skin. However, in piglets treated 24 hr or more after birth only the intestinal mucosa was coloured, and there was no evidence of transmission into the lymph nodes. These findings suggest that intestinal absorption of macromolecules in piglets is a two-stage mechanism, uptake (pinocytosis) into the epithelial cells being followed, in animals aged less than 24 hr, by transmission of the pinocytosed material into the lymphatic system.

PATHOLOGY

3121. Starch powder reprieved?

Henderson, W. J., Melville-Jones, C., Barr, W. T. & Griffiths, K. (1975). Identification of talc on surgeons' gloves and in tissue from starch granulomas. *Br. J. Surg.* **62,** 941.

Although starch powder has replaced talc as a lubricant for surgical gloves owing to the implication of talc in the aetiology of postoperative granulomas, there is much evidence (*Cited in F.C.T.* 1974, **12,** 261; *ibid* 1975, **13,** 677) to incriminate starch powder as the cause of a similar granulomatous reaction. The authors cited above report their finding of talc particles in lesions diagnosed as 'starch' granulomas and suggest that starch may stand unjustly accused in these and similar cases.

Particulate preparations were obtained from histological sections of tissue reported to be 'starch', 'foreign-body' and 'suspected talc' granulomas and from various surgeons' gloves.

Sections from six 'starch' granulomas contained significant quantities of talc particles and sections from

three 'suspected talc' granulomas contained large numbers of talc particles. In the 20 pairs of British gloves tested, a typical result showed 8000 talc particles/mm^2 of glove. No contamination was found on four pairs of Austrian gloves, but one pair of American gloves was heavily contaminated. The talc particles on the outer surface of the gloves were about 20 nm in diameter.

The authors suggest that submicroscopic particles of the talc may be concerned in foreign-body granulomas other than those diagnosed as talc granulomas.

CANCER RESEARCH

3122. More nitrosamine studies

Taylor, H. W. & Nettesheim, P. (1975). Influence of administration route and dosage schedule on tumor response to nitrosoheptamethyleneimine in rats. *Int. J. Cancer* **15**, 301.

Nitrosoheptamethyleneimine (NHMI), one of the cyclic nitrosamines, was found to be a potent carcinogen in rats when administered in drinking-water (*Cited in F.C.T.* 1974, **12**, 254). In the study cited above, rats were given NHMI either intragastrically or by sc injection, to provide information on the relationship between the dose of carcinogen and tumour incidence.

NHMI was administered intragastrically to Fischer (F-344) rats twice weekly for 20 wk at levels of 3·75, 7·5, 15 and 30 mg/kg. A single group of Sprague–Dawley rats was similarly treated with doses of 30 mg/kg. The animals were observed over their whole lifespan. In the sc experiments, only Fischer rats were used. Six groups of animals were given ten weekly injections at levels ranging from 0·55 to 40 mg/kg, three groups were treated twice weekly for 4 wk with doses of 2·8, 5·5 or 16·6 mg/kg and two groups received single injections of 44 or 133 mg/kg. All surviving animals were killed at wk 90.

None of the rats given NHMI intragastrically survived longer than 60 wk, and an inverse relationship was observed between survival time and dose of carcinogen. There were no differences in type or number of tumours in the two strains, the nasal cavity, larynx, trachea and oesophagus being the most common tumour sites at all dose levels. Squamous-cell carcinomas occurred frequently in the nasal cavity, and adenocarcinomas were observed in small numbers. The incidence of squamous-cell lung tumours was dose related.

After sc injection of NHMI for 10 wk, an inverse relation between dose and survival was again evident. This decrease in survival time was not noted at the lower dose levels, or at any of the treatment levels included in the 4-wk or single-dose régimes. There was a significant increase in tumour incidence at the two highest dose levels in the 10-wk study and at the highest level in the 4-wk study. Injection of NHMI again produced a high incidence of tumours in the nasal cavity, the incidence being dose related.

The small numbers of pulmonary tumours found after NHMI injection were classified as alveologenic adenomas and adenocarcinomas. As previous workers had suggested that the route of administration was unimportant in the tumour response to systemic carcinogens, the variation in the type of lung tumour evident in this study (adenomas and adenocarcinomas following injection and squamous cell tumours after intragastric administration) was noteworthy.

The overall incidence of tumours was much higher in the rats given the nitrosamine intragastrically, and the authors suggest that this was probably a consequence of metabolism by the liver. This study also illustrates the importance of the dose schedule in producing a carcinogenic response, the cumulative carcinogenic dose given over a 10-wk or 4-wk period being much more tumorigenic than the same dose given in a single treatment.

Fd Cosmet. Toxicol. Vol. 15, p. 89. Pergamon Press 1977. Printed in Great Britain

ANNOUNCEMENTS

ISRAELI DRUG SYMPOSIUM

Details are now available of the International Symposium on Drug Activity to be held in Jerusalem, Israel, on 27 March–1 April 1977. The symposium, organized by the School of Pharmacy, Hebrew University, Jerusalem, and sponsored by the Board of Pharmaceutical Sciences (F.I.P.) will be concerned, among other topics, with drug metabolism, particularly interspecies variation and the role of the gastro-intestinal flora, with kinetic studies of the absorption, elimination and bioavailability of drugs, and with biochemical approaches to medicinal research and development. Applications for registration should be made to The Organising Committee, P.O.B. 16271, Tel Aviv, Israel.

COURSES IN INDUSTRIAL TOXICOLOGY AND EPIDEMIOLOGY

Information has been received from Finland about two courses being arranged by the Institute of Occupational Health, Helsinki. An international course in industrial toxicology, to be held on 8–14 August 1977 at the Hanasaari Cultural Center, Helsinki, is intended for graduates in medicine, chemistry, pharmacology, technology or a related field. The other course, to be held on 15–26 August in the Institute's premises in Helsinki, is an advanced course in epidemiological methods, designed for holders of a degree in medicine, statistics, sociology, technology or a related field. The lectures in both courses will be given in English. The final date for registration for the Second International Course in Industrial Toxicology is 1 May 1977 and that for the Fourth International Advanced Course in Epidemiologic Methods is 15 March 1977. Further details of the former course may be obtained from Dr. Harri Vainio, Department of Industrial Hygiene and Toxicology, Institute of Occupational Health, Haartmaninkatu 1, 00290 Helsinki 29, Finland, and of the latter from Mr. Markku Nurminen, L.Sc., Bureau Chief, Department of Epidemiology and Biometry, Institute of Occupational Health, at the same address.

Fd Cosmet. Toxicol. Vol. 15, p. 91. Pergamon Press 1977. Printed in Great Britain

FORTHCOMING PAPERS

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

Enhancing effects of phenobarbitone and butylated hydroxytoluene on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. By C. Peraino, R. J. M. Fry, E. Staffeldt and J. P. Christopher.

On the aetiology of gastric cancer: Mutagenicity of food extracts after incubation with nitrite. By H. Marquardt, F. Rufino and J. H. Weisburger.

Absorption and metabolism of chlorinated fatty acids in triglycerides in rats. By H. M. Cunningham and G. A. Lawrence.

Absorption and distribution studies on chlorinated oleic acid and extracts of chlorinated lipid and protein fractions of flour in rats. By H. M. Cunningham and G. A. Lawrence.

Synthesis and properties of N^{ϵ}-(DL-2-amino-2 carboxyethyl)-L-lysine, lysinoalanine. By J. C. Woodard, D. D. Short, C. E. Strattan and J. H. Duncan.

Renal toxicity of N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine (lysinoalanine) in rats. By J. C. Woodard and D. D. Short.

Inhalation of talc baby powder by hamsters. By A. P. Wehner, G. M. Zwicker, W. C. Cannon, C. R. Watson and W. W. Carlton.

Evaluation of the corneal irritancy of test shampoos and detergents in various animal species. By L. L. Gershbein and J. E. McDonald.

Serum alkaline phosphatase and microsomal enzymes in the beagle dog. By R. T. Keefe, P. M. Newberne and T. Myers.

Inhibitory effect of butylated hydroxytoluene (BHT) on intestinal carcinogenesis in rats by azoxymethane. By E. K. Weisburger, R. P. Evarts and M. L. Wenk. (Short Paper)

Nitrate, nitrite, dimethylnitrosamine and *N*-nitrosopyrrolidine in some Chinese food products. By Y. Y. Fong and W. C. Chan. (Short Paper)

Effect of various dietary components on absorption and tissue distribution of orally administered inorganic tin in rats. By P. Fritsch, G. de Saint Blanquat and R. Derache. (Short Paper).

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[Contents continued]

REVIEW SECTION

REVIEWS OF RECENT PUBLICATIONS 63

BOOK REVIEWS 65

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST 69

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS 77

ANNOUNCEMENTS 89

FORTHCOMING PAPERS 91

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.

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