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Contents

Volume 18 Number 1

Volume 18 Number 2

Volume 18 Number 3

Volume 18 Number 4

Volume 18 Number 5

Volume 18 Number 6

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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Index of Authors

The Index of Authors contains the names of all authors of original papers, review articles and Letters to the Editor. For Reviews of Recent Publications and Abstracts, only the names of all the authors mentioned in the reference are given. For Articles of General Interest the names of all the authors mentioned in the text are provided, including those constituting the words *et al.*

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Index of Subjects*

*The significance of the superscripts P, M, R and L is given in paragraph 2 of the Index of Authors (p.3).

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Fd Cosmet. Toxicol. Vol. 18, pp. 1 to 5
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Research Section

SYNTHESIS OF ^{14}C-LABELLED FD & C BLUE NO. 1 (BRILLIANT BLUE FCF) AND ITS INTESTINAL ABSORPTION AND METABOLIC FATE IN RATS

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(*Received 19 June 1979*)

Abstract—[*Methylene*-^{14}C]FD & C Blue No. 1 was synthesized in eight steps from barium [^{14}C]carbonate. Female Sprague-Dawley rats were given a single dose (0·27 mg; 1·74 μCi) of the ^{14}C-labelled colouring by gavage. In bile-duct ligated rats, intestinal absorption of FD & C Blue No. 1 (estimated from urinary ^{14}C excretion, expired $^{14}CO_2$ and residual radioactivity in internal organs and tissues 96 hr after oral administration) averaged 2·05% of the dose. Mean faecal excretion was 97·28% and the total recovery of administered radioactivity was 99·38%. Intestinal absorption of [^{14}C]FD & C Blue No. 1 in intact rats averaged only 0·27% (91·69% recovery), while biliary excretion in bile-duct cannulated animals averaged 1·32% of the dose. Thin-layer chromatography of urine and bile samples revealed that about 95% of excreted radioactivity was unaltered FD & C Blue No. 1 and that about 5% was an unidentified metabolite or degradation product of FD & C Blue No. 1. The results show that FD & C Blue No. 1 is poorly absorbed from the gastro-intestinal tract, and undergoes subsequent rapid and complete biliary excretion.

INTRODUCTION

Although not widely used by itself in foods, FD & C Blue No. 1 is an important colouring for blends with other dyes to achieve green and purple shades. The triphenylmethane food colourings, including FD & C Blue No. 1, were reported by Hess & Fitzhugh (1955) to be poorly absorbed from the intestinal tract of rats; faecal dye excretion over 36 hr following a 200-mg oral dose was determined spectrophotometrically to be 96 $\pm$ 2·15%, and no dye was detected in the urine. Hess & Fitzhugh (1955) also reported that in two bile-duct cannulated dogs 0·7 and 2·8%, respectively, of a 200-mg oral dose was excreted in the bile. Similar figures have been reported by Minegishi & Yamaha (1974 & 1977) with other triphenylmethane colourings.

While these studies indicate that the intestinal absorption of FD & C Blue No. 1 is low, the spectrophotometric methods used may not have allowed its accurate determination. The object of this study was to evaluate intestinal absorption, tissue distribution, and biliary, urinary and pulmonary excretion of FD & C Blue No. 1 and its metabolites using radiolabelled dye.

EXPERIMENTAL

Preparation of ^{14}C-labelled FD & C Blue No. 1 (Brilliant Blue FCF; C.I. 42090)

The radiochemical synthesis of FD & C Blue No. 1 (*N*-ethyl-*N*-(4-[(4-{ethyl[(3-sulphophenyl)methyl]amino}phenyl) (2-sulphophenyl) [^{14}C]methylene]-2,5-cyclohexadien-1-ylidene)-3-sulphobenzenemethanaminium hydroxide inner salt, disodium salt) is outlined in Fig. 1.

o-[7-^{14}*C*]*Toluenesulphonic acid.* Barium [^{14}C]carbonate was converted in five steps into *o*-[7-^{14}C]-toluenesulphonic acid using established literature procedures. [^{14}C]Carbon dioxide was generated by treating barium [^{14}C]carbonate with 98% sulphuric acid, and was reacted with phenylmagnesium bromide to give [7-^{14}C]benzoic acid (Murray & Williams, 1958). This was reduced with lithium aluminium hydride (Nystrom & Brown, 1947) to yield [7-^{14}C]benzyl alcohol which was further reduced to [7-^{14}C]toluene using hydrogen and an acid-washed palladium-on-carbon catalyst (Kindler, Schärfe & Heinrich, 1949). The [7-^{14}C]toluene was sulphonated in 98% sulphuric acid at room temperature to give a mixture of *o*- and *p*-isomers of toluenesulphonic acid. Dilution with water led to crystallization of the major portion of the *p*-isomer. The mother liquor containing the *o*-isomer with about 15% *p*-isomer was used directly in the next step.

2-[7-^{14}*C*]*Formylbenzenesulphonic acid.* To a 15-ml, round bottomed flask fitted with a magnetic stirring bar and a condenser, were added 150 μl of an aqueous solution containing 7 mCi of *o*-[7-^{14}C]toluenesulphonic acid (~15% *p*-isomer), 1 ml 30% sulphuric acid and 100 mg manganese dioxide ore (pyrolusite; MC & B, Norwood, OH). The mixture was heated and stirred in an oil bath at 85°C for 1 hr, then neutralized and filtered through a medium-porosity sintered-glass funnel containing Celite (Johns-Manville Sales Corp., Denver, CO). The water was removed from the filtrate under vacuum. Extraction of the resulting white solid

with hot methanol, followed by filtration and evaporation of the filtrate under vacuum, gave 32·5 mg (6·92 mCi) of a solid. Thin-layer chromatographic (TLC) analysis of this solid (Quantum LQ6DF 20-cm silica-gel plates; acetic acid–acetonitrile (2:98, v/v); R_F = 0·48) and radiochromatographic scanning indicated that it contained 26% (1·76 mCi) 2-[7-^{14}C]formylbenzenesulphonic acid. The solid was chromatographed on a column containing 30 g silica gel (Merck No. 7734, E. Merck AG, Darmstadt, Federal Republic of Germany) using 25% methanol in ethyl acetate as the eluent. Combination of the appropriate fractions identified by TLC analysis resulted in 3·07 mCi of material that, by TLC and radiochromatographic analysis, was shown to contain 49% (1·5 mCi; 21% radiochemical yield) 2-[7-^{14}C]formylbenzenesulphonic acid.

Radioactivity was measured by the liquid scintillation technique using a Packard TriCarb Model 2420 spectrometer (Packard Instrument Co., Downer's Grove, IL). Data were processed using a Tektronix (Beaverton, OR) 31 calculator equipped with a digital plotter. Radiochemical purity on TLC was determined using a Packard Radiochromatogram Scanner Model 7201.

[Methylene-^{14}C]*FD & C Blue No. 1.* The partially purified 2-[7-^{14}C]formylbenzenesulphonic acid (0·07 mmol, 1·5 mCi) was combined with 0·03 mmol (5·6 mg) carrier (total 0·1 mmol), 0·195 mmol (56·8 mg) *N*-ethyl-*N*-phenylbenzylamine-3-sulphonic acid and 2 ml aqueous sulphuric acid (pH 2) in a 25-ml, round-bottomed flask that was fitted with a condenser, an argon bubbler and a magnetic stirring bar. The mixture was heated to 100°C under argon and stirred at that temperature for 18 hr. The solution was neutralized and half of it was evaporated to dryness under vacuum. The other half was reserved for future use. The dried leuco compound was dissolved in 3 ml 10% aqueous *N*,*N*-dimethylformamide and chloranil (4·92 mg, 0·02 mmol) was added. The mixture was heated in an oil bath at 100°C for 10 min and then cooled to room temperature.

The blue dye was precipitated by the addition of a solution of ether–ethanol (3:1, v/v). The precipitate was dissolved in methanol, filtered and re-precipitated with methanol–ether (2:1, v/v). The yield was 311 μCi, but HPLC analysis showed that the crude product was of low purity. Therefore the dye was purified by preparative high performance liquid chromatography as described below.

Purification of [^{14}C]*FD & C Blue No. 1 by high performance liquid chromatography (HPLC).* The [^{14}C]-FD & C Blue No. 1 was further purified using reverse-phase ion-pair preparative HPLC. Resolution of the product from other reaction-mixture components was accomplished using a volatile mobile phase.

The HPLC apparatus included two Model M-6000A pumps, a Model 660 solvent programmer and a Model U6K universal injector, all from Waters Associates, Inc., Milford, MA. The detector was a Model SF770 spectroflow monitor equipped with a Model GM770 monochromator (Schoeffel Instrument Corp., Westwood, NJ). For the HPLC preparative work a Waters Associates 30 cm long and 7·8 mm ID reverse-phase μBondapak-C_{18} column (octadecyltrichlorosilane covalently bonded to 10 μm μPorasil packing, 9000 plates/meter) was used. A linear (Program 6) gradient of 0–50% spectrograde methanol in 0·1 M-ammonium acetate (60 min, 6·0 ml/min) was used.

Aliquots (200 μl) of an approximately 0·1% solution of crude [^{14}C]FD & C Blue No. 1 were injected into the HPLC and eluent fractions were collected. The retention time of FD & C Blue No. 1 was determined by co-injection with commercially obtained material (Lot Y9185, Allied Chemical Corp., Morristown, NJ). Eleven injections were required to purify the entire reaction mixture. The purest fractions from each injection were combined, evaporated and placed under vacuum to eliminate methanol, water and ammonium acetate, and then redissolved in water.

This sample of combined fractions was injected onto an analytical C_{18} column. The resultant chromatogram indicated that further purification was necessary. Three preparative injections were made and the eluent fractions were collected. Again, the purest fractions from each injection were combined, evaporated, and diluted to 2 ml with water. A 10-μl aliquot of this

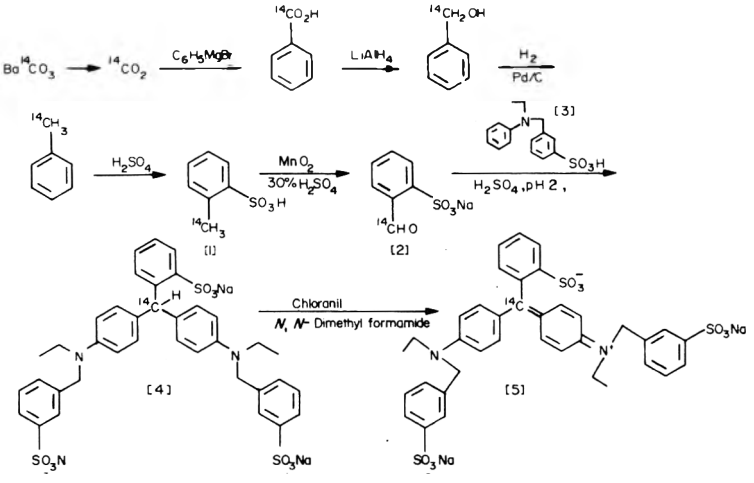

Fig. 1. Scheme of radiochemical synthesis of [*methylene*-^{14}C]FD & C Blue No. 1. [1] *o*-[7-^{14}C]-Toluenesulphonic acid. [2] 2-[7-^{14}C]Formylbenzenesulphonic acid. [3] *N*-Ethyl-*N*-phenylbenzylamine-3-sulphonic acid. [4] Dried leuco compound. [5] [*Methylene*-^{14}C]FD & C Blue No. 1.

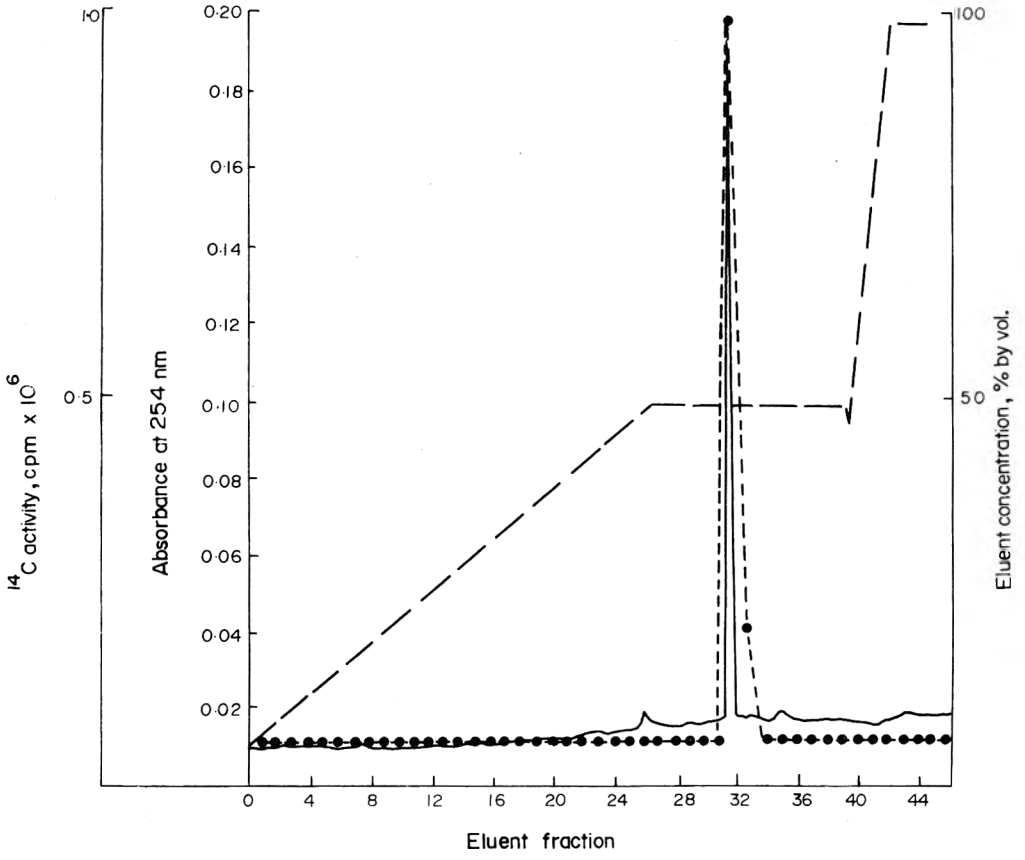

Fig. 2. HPLC purification of [^{14}C]FD & C Blue No. 1: absorbance of effluent at 254 nm (—); ^{14}C activity (---●---); eluent gradient (——).

further purified material was injected onto the analytical C_{18}-column system. Fractions of this run were collected at 2-ml intervals. These solutions were counted for radioactivity and 11% of the total radioactivity was found eluting with other than the main peak of [^{14}C]FD & C Blue No. 1. Therefore this sample needed further purification.

The sample was concentrated. Five preparative injections were made and the eluent fractions were collected. The purest fractions from each injection were combined, evaporated and diluted to 2 ml with water. A 10-μl aliquot of this approx. 1 mg/ml solution was injected onto the analytical column. Fractions were collected at 2-ml intervals and counted for radioactivity. The purity of the resultant [^{14}C]FD & C Blue No. 1 was determined to be more than 99% (Fig. 2).

Animal studies

Thirteen female Sprague–Dawley rats (175–190 g; Simonsen Laboratories, Gilroy, CA) were acclimatized to stainless-steel metabolism cages for 4 days before the study. Three of the rats were bile-duct cannulated 2 days before dosing and were housed as described previously (Enderlin & Honohan, 1977). One day before dosing, five of the rats were bile-duct ligated (Halladay, Enderlin, Parkinson & Honohan, 1978), and all the rats were fasted overnight and until 8 hr after dosing. Except during the fast, all the rats had free access to food (Simonsen white diet powder) and water throughout the study.

Each rat was given 1 ml (1·74 μCi, 0·27 mg) of the dose solution by gavage. Two rats each from the intact and bile-duct ligated groups were placed in CO_2-collection chambers immediately after dosing. The CO_2-trapping agent from these chambers was sampled after 24 and 48 hr. After 48 hr, $^{14}CO_2$ excretion was negligible, so these rats were placed in normal metabolism cages for the remainder of the study. Urine and faeces were collected from intact and bile-duct ligated rats and bile was collected from the bile-duct cannulated rats after 24, 48, 72 and 96 hr. After 96 hr, the rats were killed and samples of internal organs, tissues and gut contents were collected. All collections were prepared and assayed for radioactivity as described previously (Enderlin & Honohan, 1977; Parkinson, Honohan, Enderlin, Halladay, Hale, de Keczer, Dubin, Ryerson & Read, 1978).

Thin-layer chromatography (TLC) of urine and bile samples. Samples (3 ml) of the 24-hr urine or bile collections were pooled. The pooled samples were concentrated by evaporation under nitrogen and divided into two. One of each of the urine and bile pools was adjusted to pH 4·5 with HCl and was incubated overnight at 37°C in the presence of β-glucuronidase–sulphatase mixture (400 U/ml sample; Type 1, Sigma Chemical Co., St. Louis, MO). These treated samples plus the remaining untreated urine and bile concentrates (pH 6·5) were spotted onto silica-gel TLC plates

(Quantum LQ6DF), along with 10 μl [^{14}C]FD & C Blue No. 1. The solvent system was made by combining, in order, the following solvents in the proportions given: *n*-butanol–absolute ethanol–water–concentrated ammonia (200:45:88:2, by vol.). The plates were allowed to air dry and the resulting bands were located under visible and UV light. The silica gel was scraped from the TLC plate at intervals corresponding to the position of the located bands and each band was placed in a separate scintillation vial. (A faint blue band was visible on the chromatograms of the urine samples of the bile-duct ligated rats at approximately the R_F of FD & C Blue No. 1). The silica was extracted with 5 ml water and 0·5 ml Soluene (Packard Instrument Co.), for 4 hr and then scintillation fluid (Dimilume; Packard Instrument Co.) was added. Before counting, the vials were allowed to equilibrate overnight in a Packard TriCarb Model 3385 Scintillation Spectrometer. The results were plotted on a Tektronix Model 31 calculator with Tektronix Model 4661 X, Y plotter. The total recovery of the radioactivity applied to each plate was 80·4 ± 24·4% (mean ± SD, N = 3).

RESULTS AND DISCUSSION

Radiochemical synthesis

The synthesis of ^{14}C-labelled FD & C Blue No. 1 (Fig. 1) was based on the synthesis of Xylene Blue VS (C.I. 42045) described by Fierz-David & Blangly (1949), which uses benzaldehyde-2,4-disulphonic acid as the starting material. In a reaction analogous to that using benzaldehyde-2,4-disulphonic acid, attempts were made to prepare 2-[7-^{14}C]formylbenzenesulphonic acid from *o*-[7-^{14}C]toluenesulphonic acid using manganese dioxide and concentrated sulphuric acid, but only brown tar containing no aldehyde was produced. Various oxidants, including Seloxcette (Aldrich Chemical Co., Milwaukee, WI), cerric ammonium nitrate, chromic oxide and activated manganese dioxide (Willowbrook Labs, Waukesha, WI), were tried under a variety of conditions, but none of the desired product was produced.

The *o*-[7-^{14}C]toluenesulphonic acid was finally converted to 2-[7-^{14}C]formylbenzenesulphonic acid in 40–60% yield with pyrolusite (MnO_2 ore) by heating the mixture in 30% sulphuric acid for 1 hr. Purification by liquid chromatography was necessary to provide material sufficiently pure for the next step.

Condensation of 2-[7-^{14}C]formylbenzenesulphonic acid with *N*-ethyl-*N*-phenylbenzylamine-3-sulphonic acid was accomplished readily under conditions described by D. Lindblat (personal communication 1978) using a slight excess of the aldehyde. Oxidation of the leuco compound on this scale (0·1 mmol) proved to be somewhat difficult, as the usual procedures using lead peroxide paste or sodium dichromate proved to be unworkable. The conversion was accomplished using chloranil in 10% aqueous *N*,*N*-dimethylformamide following the procedure of Bell (1973). The crude dye was purified by precipitation from ethanol and methanol with ether and preparative HPLC.

The labelled dye produced after this exhaustive purification contained less than 1% labelled impurities. The dye (Light Green SF Yellowish, C.I. 42095) formed from the *p*-[7-^{14}C]toluenesulphonic acid present in the starting material (approx. 15%) was effectively removed by the HPLC purification. The overall yield, while very low (approx. 0·2%), provided material for absorption studies which far exceeded commercial FD & C Blue No. 1 in purity. Without HPLC techniques, material of this purity would be almost impossible to obtain.

Absorption, excretion and metabolic fate

The absorption, excretion and tissue-distribution data are given in Table 1. Urinary, faecal and pulmonary excretion of radioactivity after oral administration of [^{14}C]FD & C Blue No. 1 to bile-duct ligated rats were 2·02, 97·28 and 0·01% of the administered dose, respectively. Radioactivity recovered from internal organs represented 0·02% of that administered and total recovery of administered radioactivity was 99·38%. The estimate of total intestinal absorption based upon the radioactivity in urine, internal organs and CO_2 from bile-duct ligated rats was 2·05 ± 0·35% of the dose (mean ± SD). Intestinal absorption in intact rats averaged only 0·27% (91·69% recovery), while biliary excretion in bile-duct cannulated animals averaged 1·32% of dose.

TLC of the 24-hr urine and bile samples from bile-duct ligated and bile-duct cannulated groups respectively, showed about 95% of the radioactivity in these samples to be located at the R_F of [^{14}C]FD & C Blue No. 1 regardless of enzymatic treatment. These results indicate that [^{14}C]FD & C Blue No. 1 was excreted mostly unchanged in the bile and urine. The re-

Table 1. *Recovery of radioactivity 96 hr after oral administration of a single dose (0·27 mg; 1·74 μCi) of [^{14}C]FD & C Blue No. 1 to rats*

Percentage of administered radioactive dose recovered in							
Urine	Faeces	Organs	Gut contents	Respiratory CO_2	Bile	Cage	Total
Bile-duct ligated rats							
2·02 ± 0·35	97·28 ± 1·56	0·02 ± 0·005	0·01 ± 0·01	0·01 ± 0·00	—	0·04 ± 0·02	99·38 ± 1·62
Intact rats							
0·22 ± 0·29	91·14 ± 2·61	0·01 ± 0·01	0·08 ± 0·18	0·04 ± 0·004	—	0·20 ± 0·19	91·69 ± 2·50
Bile-duct cannulated rats							
—	—	—	—	—	1·32 ± 0·90	—	—

Values are means ± SD for groups of five rats, or, for the determinations of dose recovery in respiratory CO_2 and bile, for groups of two and three rats, respectively.

mainder of radioactivity was unidentified, but roughly corresponded with a small radioactive peak also detected by TLC of the dose solution standard. This minor radioactive peak in the dose solution increased from about 2 to about 5% during 4 months of use and may have been a decomposition product resulting from loss of a sulphonate group from the FD & C Blue No. 1 molecule (Stein, 1969).

Our results confirm earlier reports that FD & C Blue No. 1 is very poorly absorbed from the gastrointestinal tract, although following absorption it undergoes extensive biliary excretion. The relatively small degree of absorption and subsequent metabolism, coupled with the relatively rapid excretion by biliary and urinary routes, could partially explain the low oral toxicity of this and structurally related colourings (Radomski, 1974).

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THE METABOLIC DISPOSITION OF ^{14}C-LABELLED GREEN S AND BRILLIANT BLUE FCF IN THE RAT, MOUSE AND GUINEA-PIG

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Abstract—The absorption, metabolism and excretion of ^{14}C-labelled Green S and Brilliant Blue FCF have been studied in the rat, mouse and guinea-pig. Following administration of a single oral dose of Green S at either 100 μg/kg or 10 mg/kg or of Brilliant Blue FCF at either 30 μg/kg or 3 mg/kg to male or female rats, substantially all of the dose was excreted unchanged in the faeces within 72 hr. Pretreating male rats with unlabelled Green S or Brilliant Blue FCF in the diet (100 or 30 mg/kg/day, respectively) for 21 days prior to dosing with ^{14}C-labelled colouring had no effect on the route of excretion or the time taken to eliminate all of the label. Similarly male mice and guinea-pigs excreted in the faeces all of a single oral dose of Green S or Brilliant Blue FCF. The lack of absorption and metabolism of the labelled dye in the gastro-intestinal tract of all three species investigated was confirmed by studies using isolated loops of small intestine. It was also shown that no radioactivity was taken up by the foetuses of pregnant rats given ^{14}C-labelled Green S or Brilliant Blue FCF.

INTRODUCTION

A number of water-soluble triphenylmethane dyes including Green S and Brilliant Blue FCF are currently permitted for use in the UK in food and cosmetics preparations. Although a considerable body of evidence relating to the toxicology of this class of dyes is available (IARC Working Group, 1978; Joint FAO/WHO Expert Committee on Food Additives, 1970 & 1974), few published data exist on the metabolic disposition of triphenylmethane dyes.

Green S (CI (1956) No. 44090; EEC E142) is the monosodium salt of 4,4′-bis(dimethylamino)diphenylmethylene-2-naphthol-3,6-disulphonic acid. It is used primarily in canned soups and vegetables, pickles and sauces, dessert mixes and jellies, at concentrations up to 60 mg/kg. Following the oral administration of the colouring at a dose level between 250 and 400 mg/kg to rats, J. W. Daniel (unpublished data 1959, cited by Joint FAO/WHO Expert Committee on Food Additives, 1974) showed that approximately 30% of the dye was recovered in the faeces and up to 0·34% in the urine. However, he found no evidence for metabolism of the dye in this species. Dalgaard-Mikkelsen & Rasmussen (1962) fed Green S dissolved in milk at a concentration of 25 mg/litre to pigs and calves and recovered all of the administered material in the faeces within 5 days and 1–2 wk, respectively.

Brilliant Blue FCF (CI (1956) No. 42090; FD & C Blue No. 1) is the disodium or diammonium salt of 4-([4-(*N*-ethyl-*p*-sulphobenzylamino)phenyl]-(2-sulphoniumphenyl)methylene)-[1-(*N*-ethyl-*N*-*p*-sulphobenzyl)-$\Delta^{2,5}$-cyclohexadienimine]. The disodium salt is used in soft drinks, ice cream and sugar confectionary and the diammonium salt in drugs and cosmetics. Following the oral administration of between 200 and 1000 mg/kg to rats, rabbits and dogs, Hess & Fitzhugh (1953, 1954 & 1955) showed that almost all the dye was recovered unchanged in the faeces, with none appearing in the urine. The latter paper reported some biliary excretion of dye from orally-dosed animals, with a maximum excretion of 2·8% of the dose from the dog. J. W. Daniel (unpublished data 1959, cited by Joint FAO/WHO Expert Committee on Food Additives, 1970) also found no urinary excretion of dye after giving an oral dose of 400 mg/kg to rats, although after sc administration approximately 2·5% of the dose was recovered in the urine.

The results presented in this report describe the absorption, distribution and excretion of ^{14}C-labelled Green S and Brilliant Blue FCF in the rat, mouse and guinea-pig following oral administration of dose levels of 100 μg and 10 mg Green S/kg and 30 μg and 3 mg Brilliant Blue FCF/kg.

EXPERIMENTAL

Materials. Green S, labelled with ^{14}C in the central methane group and uniformly in the benzene ring (Fig. 1a), and Brilliant Blue FCF labelled with ^{14}C in the central methane group (Fig. 1b) were supplied by ICI Physics and Radioisotopes Services, Cleveland. The Green S had a specific activity of 13·6 mCi/mmol (mol wt 576·6) and a radiochemical purity of >97% by thin-layer chromatography on Silicagel G. The Brilliant Blue FCF had a specific activity of 16·2 mCi/mmol (mol wt 794) and a radiochemical purity of >95% by thin-layer chromatography. Chromatograms were developed in either butan-2-one–acetone–H_2O (8:8:2, by vol.), ethyl acetate–methanol–NH_3 0·88 sp. gr. (6:3:1, by vol.) or butan-2-one–acetone–isopropanol–H_2O (3:3:3:1, by vol.) (Figs 2a,b). Unlabelled colourings were supplied by the Food Colours Committee of the Chemical Industries Association and complied with The Colouring

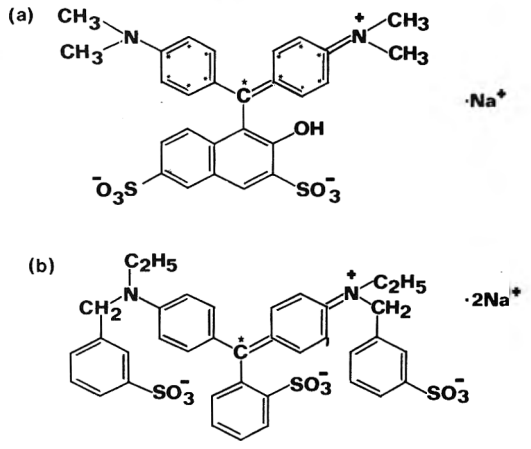

Fig. 1. Structures of (a) Green S and (b) Brilliant Blue FCF, with ^{14}C-labelled positions indicated by an asterisk.

Matter in Food Regulations 1973 (Statutory Instrument 1973 no. 1340). Precoated silicagel GFHL plates were obtained from Anachem Ltd., Luton, Beds.

Animals and dosing. The studies were carried out in male and female Wistar albino rats (120–150 g body weight), male Tuck TO strain mice (30–50 g body weight) and male Dunkin-Hartley guinea-pigs (250–350 g body weight) supplied by OLAC (1976) Ltd., Bicester, Oxon. The rats and mice were maintained on Spillers Laboratory Small Animal Diet No. 1 and the guinea-pigs on Oxoid Diet SG1 with a vitamin C supplement in the drinking-water. All animals were given food and water *ad lib.* and kept at 20 ± 1°C. For *in vivo* experiments, the ^{14}C-labelled colouring was administered by oral gavage in aqueous solution (5 ml/kg body weight) at a dose level of either 100 μg/kg or 10 mg/kg for Green S or 30 μg/kg or 3 mg/kg for Brilliant Blue FCF. In the experiments involving predosing, unlabelled colouring was mixed with the diet and administered for 21 days to provide a dose level of either 100 mg/kg/day for Green S or 30 mg/kg/day for Brilliant Blue FCF. For intestinal absorption studies, ^{14}C-labelled colouring, diluted where appropriate with unlabelled material, was dissolved in 0·9% (w/v) saline to give solutions of 10, 100 and 1000 ppm.

In vivo *metabolic studies.* Following the administration of ^{14}C-labelled colouring (between 0·6 and 6 μCi/kg body weight) animals were housed in all-glass metabolism cages (Jencons Scientific Ltd., Hemel Hempstead, Herts.). Air was drawn through the cage at a constant rate of 250 ml/min and the exhaled CO_2 was trapped in ethanolamine–2-ethoxyethanol (1:4, v/v). The trapping solutions were changed at intervals up to 72 hr. Urine and faeces were collected at 24-hr intervals for 3 days, after which time the animals were killed. In preliminary experiments the gastro-intestinal tract and major organs were removed for the determination of radioactivity.

Faecal excretion. Faeces of rats and guinea-pigs were extracted with *tert*-butanol as described by Hess & Fitzhugh (1955). The extracts were concentrated by evaporation at reduced pressure and were examined by thin-layer chromatography.

Biliary excretion. Male rats were anaesthetized with sodium pentobarbitone and the bile duct was cannulated. The ^{14}C-labelled colouring was introduced into the stomach (10 mg/kg body weight for Green S and 3 mg/kg body weight for Brilliant Blue FCF) and bile was collected for 5 hr. Total radioactivity excreted in the bile was determined.

Transplacental migration studies. Pregnant rats (200–250 g body weight) were given a single oral dose of ^{14}C-labelled Green S (10 mg/kg) or ^{14}C-labelled Brilliant Blue FCF (3 mg/kg) on day 8 of pregnancy and urine and faeces were collected at 24-hr intervals for 3 days. The animals were killed on day 11 of pregnancy and the level of radioactivity in the foetuses was determined.

Intestinal-absorption studies. Absorption was studied using the techniques described by Matthews, Craft, Geddes, Wise & Hyde (1968). Animals were

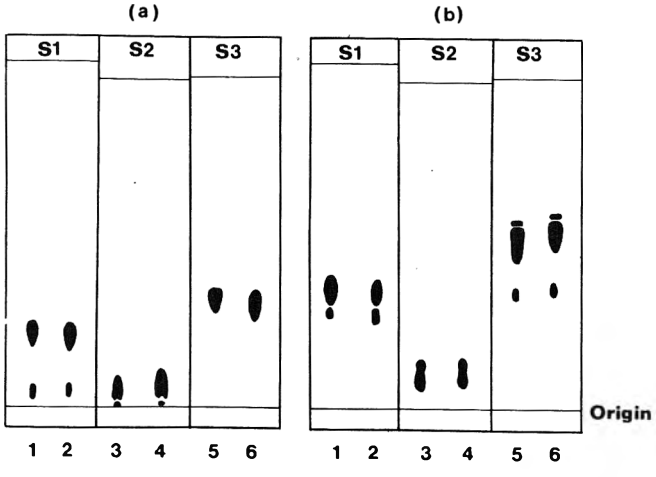

Fig. 2. Thin-layer chromatograms of (a) Green S and (b) Brilliant Blue FCF, developed with the solvent systems butan-2-one–acetone–H_2O, 8:8:2 by vol. (S1), ethyl acetate–methanol–NH_3 (0·88 sp. gr.), 6:3:1 by vol. (S2) and butan-2-one–acetone–isopropanol–H_2O, 3:3:3:1 by vol. (S3). For both colourings, tracks 1, 3 and 5 are unlabelled dye, and tracks 2, 4 and 6 are ^{14}C-labelled dye.

anaesthetized with sodium pentobarbitone and a length of small intestine was isolated between ligatures. Three separate 5-cm lengths were isolated within this length and 0·3 ml of a solution containing ^{14}C-labelled colouring and with a total concentration of labelled and unlabelled colouring of 10,100 and 1000 ppm was introduced into each loop. After absorption periods of up to 1 hr, the loops were excised and washed out with 0·9% w/v saline solution. The loop and the combined contents and washings were assayed for radioactivity.

Radioactivity determinations. Radioactivity was measured in a Packard 2650 liquid scintillation counter and efficiency was determined by the external channels-ratio method. Urine (0·5 ml), bile (0·1 ml) and intestinal-loop contents (0·5 ml) were counted in a scintillation fluid of toluene–2-ethoxyethanol (1:1, v/v) containing 2,5-diphenyloxazole (0·4%, w/v). Faeces and tissues were oxidized in a Packard 306 Sample Oxidiser and the $^{14}CO_2$ produced was trapped in Carbosorb and counted in Permafluor V (Packard Instruments, Des Plaines, IL, USA). Recovery of ^{14}C was between 97 and 99%. Radioactivity on thin-layer plates was visualized using a Radiochromatogram Spark Chamber (Birchover Instruments, Letchworth, Herts.) and was quantitated by scintillation counting of the silicagel scraped from sections of the plate.

RESULTS

After oral administration of ^{14}C-labelled Green S to male rats at dose levels of 100 μg/kg and 10 mg/kg and to female rats at 10 mg/kg and of ^{14}C-labelled Brilliant Blue FCF to male rats at dose levels of 30 μg/kg and 3 mg/kg and to female rats at 3 mg/kg, radioactivity was rapidly eliminated in the faeces, substantially all of the dose being accounted for within 72 hr (Table 1). No radioactivity was detected in expired CO_2 and a very small percentage of the dose was found in the urine (less than 2% after Green S and 0·5% after Brilliant Blue FCF). The radioactivity in the urine could have been due to leaching of the water-soluble dye from faecal material during separation in the metabolic chamber. Less than 0·01% of the dose of either colouring was associated with the gastro-intestinal tract of these animals at 72 hr and no radioactivity was detected in the liver or kidneys of the rats at autopsy. Similar results were obtained following administration of a single oral dose of ^{14}C-labelled dye to male mice and guinea-pigs (Table 2).

Treating male rats with unlabelled colouring in the diet for 21 days prior to dosing with the ^{14}C-labelled dye had no effect on the route of excretion of radioactivity or the rate of elimination (Table 3). Similarly, pregnant rats excreted substantially all of the administered radioactivity in the faeces within 72 hr (Table 4) and only 0·0001–0·0004% of the dose of Green S and 0·0004–0·0006% of the dose of Brilliant Blue FCF was detected in the total foetuses in each litter.

Thin-layer chromatography of the radioactive material extracted from the faeces of rats and guinea-pigs given a single oral dose of ^{14}C-labelled dye revealed a distribution of label similar to that of the original dye (Figs 3a,b), showing that the dye was not metabolized during its passage through the gastro-intestinal tract. The lack of absorption of the radioactive material from the intestinal tract was confirmed by studies using isolated loops of small intestine in rats, mice and guinea-pigs (Table 5). No significant absorption over a 1-hr period was found for dye concentrations ranging from 10 to 1000 ppm.

The total radioactivity excreted in the bile during the 5 hr following administration of an oral dose of ^{14}C-labelled dye to rats was also determined, and was found to be less than 0·15% of the radioactivity administered with Green S and less than 0·05% of the Brilliant Blue FCF dose.

Table 1. *Excretion of radioactivity in urine and faeces of rats given a single oral dose of ^{14}C-labelled Green S or Brilliant Blue FCF*

Route of excretion	Time (hr)	Recovery of administered radioactivity (%)		
		Males		Females
		Lower dose*	Higher dose*	Higher dose*
Green S				
Faeces	0–24	68·8 (19·2–95·3)	77·1 (70·0–83·2)	81·3 (79·8–83·0)
	24–48	24·8 (6·5–66·1)	16·4 (6·2–41·6)	13·5 (11·5–14·9)
	48–72	1·6 (0·3–3·7)	2·5 (0·7–4·5)	2·7 (0·70–4·5)
Urine	0–72	1·47 (0·78–3·76)	0·97 (0·66–1·30)	0·85 (0·8–0·9)
Faeces + urine	0–72	96·7 (88·7–115·8)	97·0 (83·3–121·8)	98·3 (94·6–101·2)
Brilliant Blue FCF				
Faeces	0–24	80·7 (57·8–98·1)	79·0 (58·6–90·9)	70·4 (63·7–80·1)
	24–48	12·7 (5·2–33·7)	15·2 (2·4–31·8)	22·8 (9·6–30·5)
	48–72	6·5 (0·1–21·7)	1·2 (0·04–2·9)	2·8 (0·7–5·1)
Urine	0–72	0·38 (0·21–0·67)	0·25 (0·08–0·58)	0·11 (0·06–0·18)
Faeces + urine	0–72	100·2 (87·4–115·5)	95·7 (85·8–105·6)	96·1 (94·7–97·5)

*The lower doses were 0·1 mg/kg for Green S and 0·03 mg/kg for Brilliant Blue FCF and the higher were 10 and 3 mg/kg, respectively.

Results are expressed as the means for groups of five male or four female rats, with the range of values in brackets.

Table 2. *Excretion of radioactivity in urine and faeces of male mice and guinea-pigs given a single oral dose of ^{14}C-labelled Green S or Brilliant Blue FCF*

Route of excretion	Time (hr)	Recovery of administered radioactivity (%)			
		Mouse		Guinea-pig	
		Lower dose*	Higher dose*	Lower dose*	Higher dose*
		Green S			
Faeces	0–24	93·3 (85·0–99·2)	93·3 (84·7–106·3)	50·4 (38·8–62·9)	63·7 (53·9–70·8)
	24–48	3·6 (0·2–7·2)	0·7 (0·0–2·9)	36·9 (32·9–40·6)	31·0 (24·8–36·3)
	48–72	0·1 (0·0–0·3)	0·7 (0·03–1·9)	7·2 (3·8–10·4)	4·2 (2·4–6·5)
	72–96	ND	ND	1·8 (0·6–3·2)	ND
Urine	0–72	1·69 (0·002–2·54)	1·90 (0·36–3·57)	0·61 (0·21–1·03)	1·26 (1·03–1·49)
Faeces + urine	0–72	98·8 (94·5–102·9)	97·4 (87·4–108·7)	94·3 (85·5–100·8)†	99·8 (97·2–109·9)
		Brilliant Blue FCF			
Faeces	0–24	81·9 (74·3–88·0)	49·8 (45·5–52·7)	65·0 (52·7–73·8)	61·9 (55·0–67·4)
	24–48	11·1 (2·5–24·0)	54·1 (41·2–48·0)	27·6 (20·7–36·8)	34·1 (31·3–37·7)
	48–72	2·5 (0·3–6·0)	0·2 (0·001–0·6)	4·3 (1·0–8·2)	2·0 (0·01–5·6)
Urine	0–72	0·92 (0·67–1·15)	0·02 (0·01–0·04)	0·76 (0·25–1·10)	0·47 (0·17–0·73)
Faeces + urine	0–72	96·4 (90·8–101·5)	95·1 (86·8–101·1)	97·6 (90·0–103·5)	98·5 (96·9–99·6)

ND = Not determined

*The lower doses were 0·1 mg/kg for Green S and 0·03 mg/kg for Brilliant Blue FCF and the higher were 10 and 3 mg/kg respectively.

†Faecal excretion, 0–96 hr plus urinary excretion, 0–72 hr.

Results are expressed as the means for groups of six mice or guinea-pigs for Green S and three mice or six guinea-pigs for Brilliant Blue FCF, with the range of values in brackets.

Table 3. *Excretion of radioactivity in urine and faeces of male rats given an oral dose of either ^{14}C-labelled Green S (10 mg/kg) or ^{14}C-labelled Brilliant Blue FCF (3 mg/kg) following 21 days feeding with unlabelled dye*

Route of excretion	Time (hr)	Recovery of administered dose (%)	
		Green S	Brilliant Blue FCF
Faeces	0–24	67·4 (32·5–87·0)	65·9 (54·2–84·4)
	24–48	26·0 (8·3–66·9)	29·5 (12·9–43·6)
	48–72	0·8 (0·6–0·9)	1·0 (0·4–1·6)
	0–72	94·2 (89·8–100·7)	96·4 (94·2–99·9)
Urine	0–72	1·05 (0·88–1·15)	0·63 (0·36–0·88)
Faeces + urine	0–72	95·3 (90·7–102·1)	97·0 (94·6–100·5)

Results are expressed as the means for four animals, with the range of values in brackets.

Table 4. *Excretion of radioactivity in urine and faeces of pregnant rats given a single oral dose of ^{14}C-labelled Green S (10 mg/kg) or Brilliant Blue FCF (3 mg/kg) on day 8 of pregnancy*

Route of excretion	Time (hr)	Recovery of administered dose (%)	
		Green S	Brilliant Blue FCF
Faeces	0–24	84·1 (69·0–94·6)	72·9 (44·6–94·2)
	24–48	12·4 (2·8–24·5)	22·1 (2·0–49·6)
	48–72	0·4 (0·2–0·8)	2·4 (0·3–5·2)
Urine	0–72	0·11 (0·05–0·16)	0·12 (0·03–0·32)
Faeces + urine	0–72	97·0 (86·3–105·2)	97·5 (92·7–102·1)

Results are expressed as the means for five animals, with the range of values in brackets.

Table 5. *Absorption of ^{14}C-labelled Green S or Brilliant Blue FCF from isolated loops of small intestine of mice, rats and guinea-pigs*

Species	Time (hr)	Initial concn (ppm)...	Retained radioactivity (% of injected dose)		
			10	100	1000
			Green S		
Mouse	0		94·4 ± 4·4 (6)	96·5 ± 3·8 (6)	98·4 ± 3·7 (4)
	1		104·0 ± 5·6 (6)	98·8 ± 2·3 (6)	96·6 ± 2·7 (5)
Rat	0		96·7 ± 4·7 (4)	94·9 ± 2·4 (5)	94·7 ± 7·2 (4)
	1		94·1 ± 5·4 (6)	100·0 ± 3·3 (6)	97·7 ± 1·2 (6)
Guinea-pig	0		95·5 ± 6·3 (6)	90·8 ± 3·6 (4)	95·3 ± 4·0 (6)
	1		89·9 ± 0·7 (2)	94·2 ± 4·5 (6)	94·9 ± 3·2 (6)
			Brilliant Blue FCF		
Mouse	0		97·4 ± 3·8 (6)	95·3 ± 3·2 (6)	94·5 ± 8·0 (6)
	1		102·1 ± 3·5 (5)	95·4 ± 13·1 (6)	88·1 ± 6·3 (6)
Rat	0		99·6 ± 6·8 (4)	96·9 ± 2·3 (6)	94·5 ± 1·3 (6)
	1		99·3 ± 4·5 (6)	98·9 ± 6·1 (9)	87·5 ± 1·3 (6)**
Guinea-pig	0		101·0 ± 4·2 (6)	94·1 ± 7·4 (6)	92·8 ± 2·3 (6)
	1		91·8 ± 3·8 (6)**	87·6 ± 7·2 (4)	92·5 ± 4·6 (6)

Results are expressed as means ± 1 SD, with the number of determinations indicated in brackets. Values marked with asterisks are significantly less (**P <0·01 by Student's t test) than the corresponding zero-time values.

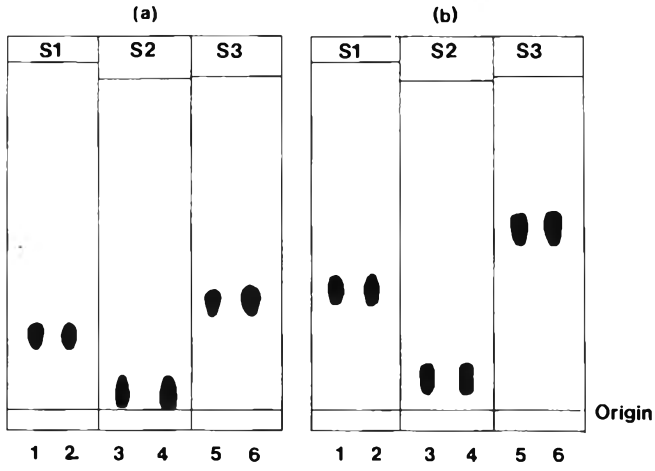

Fig. 3. Thin-layer radiochromatograms of (a) Green S and (b) Brilliant Blue FCF, developed with the solvent systems butan-2-one–acetone–H_2O, 8:8:2 by vol. (S1), ethyl acetate–methanol–NH_3 (0·88 sp. gr.), 6:3:1 by vol. (S2) and butan-2-one–acetone–isopropanol–H_2O, 3:3:3:1 by vol. (S3). Tracks 1, 3 and 5 are ^{14}C-labelled dye, and tracks 2, 4 and 6 are *tert*-butanol extracts of rat faeces.

DISCUSSION

Early work by Hess & Fitzhugh (1955) and by J. W. Daniels (unpublished data 1959, *loc. cit.*) on the absorption and metabolism of a number of unlabelled triphenylmethane dyes, including Brilliant Blue FCF and Green S, suggested that in the rat and dog there was no significant absorption or biotransformation of orally administered material at dose levels of 0·2–2 g/kg. However, although these authors found no unchanged dye or possible metabolites in the urine (for example, 2-naphthol-6,8-disulphonic acid after Green S administration), the recovery of dye from the faeces was incomplete, only 30% of a dose of Green S between 89 and 96% of a dose of Brilliant Blue FCF being recovered. In addition, Hess & Fitzhugh (1954 & 1955) reported that small amounts (less than 5% of the dose) of some of the dyes, including Brilliant Blue FCF, were excreted unchanged in the bile of rats, rabbits and dogs, indicating some measure of gastro-intestinal absorption.

Our results with ^{14}C-labelled Brilliant Blue FCF and Green S show that these dyes are not absorbed when given orally to rats, mice or guinea-pigs at dose levels of the order of 30 μg/kg to 10 mg/kg, and are not metabolized in the gastro-intestinal tract of these species. They also demonstrate that, in the rat, prolonged exposure to the dyes at the higher dose levels has no significant effect on the biological fate of the compounds. Furthermore, the fate of both dyes is similar in pregnant and virgin rats, negligible amounts of radioactivity being taken up by the foetuses. The lack of absorption of the dyes was confirmed by our finding that less than 0·15% of a dose of ^{14}C-labelled Green S and less than 0·05% of a dose of ^{14}C-labelled Brilliant Blue FCF was excreted in rat bile over 5 hr. It is possible that this represents a trace amount of radioactive impurity in the labelled dye materials.

It seems likely, therefore, that the lack of absorption and metabolism of Green S and Brilliant Blue FCF following ingestion in the rat, mouse and guinea-pig may account for the very low order of toxicity, both acute and chronic, reported for these materials (Hansen, Fitzhugh, Nelson & Davis, 1966; Lu & Lavallée, 1964; Mannell & Grice, 1964; Mannell, Grice & Allmark, 1962; I. R. Rowland, I. F. Gaunt, J. Hardy, I. S. Kiss and K. R. Butterworth, personal communication, 1975).

No data are available on the metabolic fate of triphenylmethane dyes in man. However, the principal factors governing the absorption of the dyes in both man and experimental animals are the degree of ionization of the molecule in the gut and lumen and the extent and nature of the degradation effected by gut flora. Although the gut flora of man is both qualitatively and quantitatively different from that of the rat, mouse or guinea-pig, the gastro-intestinal tract of man contains no major groups of organisms absent from these three species (Drasar, Hill & Williams, 1970). Furthermore, the pH of the various regions of the normal human gut is substantially similar to that of the rat (Crampton, 1970) and it would be reasonable, therefore, to expect that the biological fate of the dyes would be similar in man and in the species investigated.

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Fd Cosmet. Toxicol. Vol. 18, pp. 15 to 20

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THE EFFECTS OF BUTYLATED HYDROXYANISOLE AND BUTYLATED HYDROXYTOLUENE ON RENAL FUNCTION IN THE RAT. I. EFFECTS ON FLUID AND ELECTROLYTE EXCRETION

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Abstract—The effects of the food antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on renal function were studied. Male rats were given 500 mg BHA or BHT/kg/day by gavage for 6 days. Control animals received corn oil alone. Animals were offered a grain diet and distilled water *ad lib.* Water and food intakes, and urinary volume, osmolality, and sodium and potassium concentrations were monitored daily for each animal. Following the second dose of BHT, the urine volume of treated animals was increased by 78% while water intake was reduced 35%. No discrepancies occurred in the fluid balances of BHA-treated or control animals. The osmolality of urine from animals receiving BHT was reduced for the 6 days of the experiment; that of BHA and control groups was unaffected. Urinary sodium and potassium concentrations were reduced in animals treated with either antioxidant. On the second day of BHT administration, excretion of electrolytes was reduced, but not in proportion to food intake. This may have been related to hormonal changes accompanying the depression of food intake. Total daily sodium excretion of BHA-treated animals was less than expected from food intake on days 2–6, possibly owing to interference with renal prostaglandin synthesis.

INTRODUCTION

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are synthetic antioxidants added to fats and oils to prevent rancidity. Little information is available regarding the effects of BHA and BHT on renal function although the main route of elimination is the urine (Hathway, 1966). The kidneys of man receive a large proportion of the cardiac output, 25%, in relation to their size, 0·5% of body weight (Valtin, 1973). During the process of urinary excretion, the concentration of xenobiotics may increase in the nephron as a result of the reabsorption of water, and possibly through the action of systems that actively transport organic ions from the blood into the urine. Consequently, the renal tubules may be exposed to significant concentrations of chemicals that may be in the plasma at low concentrations (Foulkes & Hammond, 1975).

Although histological changes have not been observed in the kidneys from animals treated with either antioxidant (Brown, Johnson & O'Halloran, 1959; Deichman, Clemmer, Rakoczy & Bianchine, 1955), several authors have reported impairment of urinary concentrating ability in association with administration of BHA or BHT. Increased urination by various species of laboratory animals has been noted following fatal doses of BHT (Deichman *et al.* 1955). Administration of 500 mg BHA or BHT/kg/day to rabbits resulted in markedly increased daily sodium and potassium excretion, even though the animals had stopped eating (Denz & Llaurado, 1957). Urinary aldosterone excretion was also elevated; the authors suggested that this represented hypersecretion in compensation for a tubular lesion which rendered the nephron incapable of reabsorbing sodium. It should be noted that the rabbits in the study were afflicted with an inflammatory disease of the kidneys before the experiments began. This may have resulted in greater sensitivity to stresses such as large doses of xenobiotics.

The present experiments were undertaken to investigate the effects of BHA and BHT treatments on renal function, specifically electrolyte excretion and renal concentrating ability. The dose selected is nonlethal but is known to produce histological and biochemical changes in other organs (Allen & Engblom, 1972; Witschi & Côté, 1976).

EXPERIMENTAL

Materials. The test compounds BHA and BHT were purchased from Sigma Chemical Company, St. Louis, MO.

Animals, diet and treatments. Male Sprague-Dawley rats (Spartan Research Farms, Haslett, MI) weighing 275–299 g were individually housed in stainless steel metabolism cages and given a grain diet (23·4% protein, 3·0% fat, 53·5% carbohydrate; Schemmel, Mickelsen & Motawi, 1972) and distilled water *ad lib.* The animals were allowed to adjust to the cages for 3–4 days. The day immediately preceding initiation of treatments was designated day 0. Water and food intakes were monitored daily for each rat.

BHA or BHT was administered by gavage for 6

days at a dose of 500 mg/kg/day. Control animals received a similar volume (5 ml/kg) of corn-oil vehicle.

Urine analysis. The urinary outputs of each animal were collected over 24-hour periods. At the end of each period, the volume of the samples was recorded and 5-ml aliquots were frozen in plastic test tubes. At a later date the samples were thawed, centrifuged, and sodium and potassium concentrations assayed by flame photometry (Instrumentation Laboratories, Inc., Model 143). The osmolality of each sample was determined by freezing point depression (Advanced Instruments Osmometer, Model 3L).

Statistical analysis. The data are expressed as the mean ± SEM of four values. The data were subjected to analyses of variance and the differences among the means were tested with Duncan's multiple range test, using $P < 0.05$ as the criterion of significance (Steel & Torrie, 1960).

RESULTS

Food intake

The food intakes of control animals were depressed by approximately 15% for each of the 6 days of the experiment (Table 1); the average dose of corn oil represented about 11% of the normal caloric intake from the grain diet (Schemmel *et al.* 1972). Following one dose of either antioxidant, the food intake of treated animals was depressed about 38% compared with control (corn oil) rats. With continued BHT treatment the food intake decreased further, reaching a minimum following the second dose. The food intakes of animals receiving BHA or BHT subsequently returned to control values by day 6.

Fluid balance

No significant changes occurred during the course of the experiment in either the water intake or urine volume of the control or BHA-treated animals (Table 2). There was a large increase in urine volume in the 24 hr following the second dose of BHT, compared to the volume on day 0. No significant changes in water intake were apparent when compared with intake at day 0, although a significant increase was evident between days 2 and 4.

Urine composition

The osmolality of the urine from BHT-treated animals was depressed on days 1 through 6; no effect was found with BHA or corn oil (Table 3). The sodium and potassium concentration in the urine of control animals did not change significantly during the 6 days of the study (Table 3). The concentration of sodium in the urine of BHA-treated animals decreased, reaching a minimum after the second dose. The concentration in the urine of animals receiving BHT was reduced to a greater degree. As with BHA, the sodium concentration increased following the fourth dose of BHT. The potassium concentration in the urine of animals treated with BHA did not change significantly during the period of treatment; that of BHT-treated animals was depressed, with a minimum on day 2.

The total daily excretion of osmotically active substances in the urine (urine volume × osmolality) decreased following one and two doses of either antioxidant (Table 4), but approached control values by day 4. The daily sodium and potassium excretion of animals receiving corn oil did not change significantly for the duration of the experiment. The potassium excretion of the BHA-treated rats was significantly reduced after one dose, and subsequently increased. Administration of BHT resulted in depressed potassium excretion on days 1 and 2. Sodium excretion was reduced significantly by treatment with either agent on days 1, 2 and 4, and by BHA on day 6.

The rats in this study were offered distilled water and were housed in wire-bottomed stainless steel cages which minimized faecal contamination of urine. The major source of sodium and potassium was assumed to be the grain ration. Therefore with adequate renal response the excretions of these electrolytes should be proportional to changes in food consumption. Throughout the study the daily urinary excretion of electrolytes by animals receiving corn oil alone was appropriate for the food intake, except for a slight excess of excretion of osmotically active substances on day 6 (Fig. 1). Following one dose of either antioxidant, the excretion of electrolytes was depressed to the same extent as the food intake. However, on day 2 of BHT treatment the total osmolal excretion, as well as potassium loss, was significantly greater than expected from food intakes. This effect was eliminated by day 6 despite continued administration of BHT.

The excretions of potassium and total osmotically active substances were concordant with food intakes in the BHA-treated animals (Fig. 1). However, the daily sodium output was significantly less than expected on days 2 and 4 of treatment.

DISCUSSION

The ability to maintain sodium and potassium balances following administration of either antioxidant was monitored in these experiments. In contrast to the results of Denz & Llaurado (1957) the daily excretion of electrolytes by animals receiving BHA or

Table 1. *Food intakes of animals receiving BHA or BHT (500 mg/kg/day) or corn oil (5 ml/kg/day)*

Treatment	Day of treatment...	Food intake (g/24 hr)				
		0	1	2	4	6
BHA		23·0 ± 1·5	13·6 ± 3·1*	16·2 ± 3·6	20·6 ± 1·5	20·6 ± 1·5
BHT		23·8 ± 1·4	13·2 ± 4·2*	9·0 ± 3·9*	18·0 ± 2·9	17·0 ± 3·8
Corn oil		25·2 ± 1·6	21·2 ± 0·7	23·0 ± 0·8	22·0 ± 0·9	20·6 ± 2·7

Values are means ± SEM for groups of at least four animals and those marked with asterisks differ significantly (Duncan's multiple range test) from the control value (*$P < 0.05$).

Table 2. *Urine volume and water intake of animals receiving BHA or BHT (500 mg/kg/day) or corn oil (5 ml/kg/day)*

Treatment	Day of treatment...	Urine volume (ml/24 hr)				Water intake (ml/24 hr)			
		0	1	2	4	0	1	2	4
BHA		15·9 ± 2·4	10·5 ± 1·8	13·8 ± 0·9	11·6 ± 0·8	38·4 ± 3·7	33·5 ± 4·6	44·3 ± 5·8	38·3 ± 2·8
BHT		14·3 ± 1·7	11·8 ± 1·2	25·5 ± 3·7*	24·7 ± 2·9*	33·8 ± 0·8	24·3 ± 4·4	22·0 ± 4·3	40·3 ± 8·2†
Corn oil		11·8 ± 1·1	10·3 ± 1·0	12·0 ± 1·3	12·7 ± 1·1	31·5 ± 2·9	29·5 ± 2·0	31·0 ± 2·2	27·0 ± 9·0

Values are means ± SEM for groups of four animals and those marked with superscripts differ significantly (Duncan's multiple range test) from the day 0 value (*$P < 0.05$) or from the day 2 value (†$P < 0.05$).

Table 3. *Concentrations of electroytes in urine of rats treated with BHA or BHT (500 mg/kg/day) or corn oil (5 ml/kg/day)*

Treatment	Day of treatment...	Concentration of electrolytes				
		0	1	2	4	6
				Urinary osmolality (mosmol/litre)		
BHA		1620 ± 79	1856 ± 396	1327 ± 186	1604 ± 87	1596 ± 47
BHT		1502 ± 169	784 ± 224*†	453 ± 88*†	908 ± 158†	692 ± 160*†
Corn oil		1647 ± 231	1788 ± 188	1755 ± 138	1692 ± 336	1825 ± 62
				Urinary Na^+ (mequiv./litre)		
BHA		143·7 ± 11·4	94·8 ± 8·3*	64·3 ± 18·3*†	82·2 ± 11·3*†	97·9 ± 11·6*†
BHT		124·4 ± 17·1	49·9 ± 15·3*†	28·3 ± 8·1*†	54·5 ± 20·1*†	73·0 ± 8·2%†
Corn oil		143·8 ± 17·7	116·7 ± 15·5	136·5 ± 16·0	165·4 ± 26·2	157·7 ± 23·8
				Urinary K^+ (mequiv./litre)		
BHA		259·3 ± 6·8	202·0 ± 29·7	183·8 ± 46·2	216·6 ± 17·8	203·6 ± 12·3
BHT		213·8 ± 35·3	108·0 ± 33·5*†	64·4 ± 14·5*†	102·3 ± 21·7*†	137·4 ± 11·1*†
Corn oil		249·4 ± 28·0	261·0 ± 32·3	248·8 ± 35·4	256·3 ± 39·6	249·4 ± 29·0

Values are means ± SEM for groups of at least four animals and those marked with superscripts differ significantly (Duncan's multiple range test) from the day 0 value (*$P < 0.05$) or from the corresponding control value (†$P < 0.05$).

Table 4. *Daily excretion of electrolytes by animals treated with 500 mg/kg/day BHA or BHT*

Treatment	Day of treatment ...	Daily excretion of electrolytes (concn in urine × urine volume in 24 hr)				
		0	1	2	4	6
				Osmol excretion (mosmol/day)		
BHA		21·8 ± 3·5	14·1 ± 1·8*	15·6 ± 3·4	19·5 ± 2·3	18·7 ± 2·8
BHT		21·8 ± 1·5	13·8 ± 1·6*	13·5 ± 2·3*†	23·8 ± 3·0	19·2 ± 2·3
Corn oil		22·3 ± 1·1	20·7 ± 1·7	22·0 ± 1·9	23·4 ± 1·2	24·3 ± 2·0
				Na^+ excretion (mequiv./day)		
BHA		2·098 ± 0·306	1·073 ± 0·233*	1·005 ± 0·306*†	1·103 ± 0·177*†	1·394 ± 0·297†
BHT		1·912 ± 0·176	0·908 ± 0·200*	0·859 ± 0·255*†	1·399 ± 0·466†	1·745 ± 0·138
Corn oil		2·150 ± 0·169	1·526 ± 0·147	2·005 ± 0·109	2·168 ± 0·136	2·236 ± 0·141
				K^+ excretion (mequiv./day)		
BHA		3·150 ± 0·417	2·350 ± 0·326*	3·147 ± 0·377	2·889 ± 0·342	2·810 ± 0·429
BHT		3·221 ± 0·273	1·933 ± 0·302†	1·974 ± 0·386†	2·702 ± 0·483	2·980 ± 0·472
Corn oil		3·727 ± 0·217	3·348 ± 0·137	3·479 ± 0·249	3·379 ± 0·268	3·624 ± 0·233

Values are means ± SEM for groups of at least four animals and those marked with superscripts differ significantly (Duncan's multiple range test) from the day 0 value (*$P < 0·05$) or from the corresponding control group value (†$P < 0·05$).

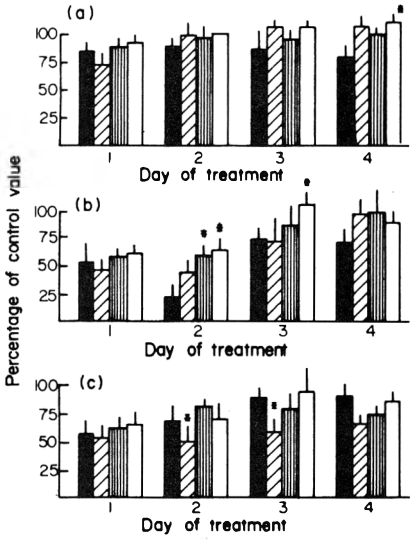

Fig. 1. Daily urinary excretion of sodium (▨), potassium (▥), and osmotically active particles (□), compared with food intake (■). The animals were given (a) corn oil (5 ml/kg/day) alone, (b) BHT (500 mg/kg/day) or (c) BHA (500 mg/kg/day). Each bar represents the mean ± SEM of at least four animals. Urinary values marked with an asterisk differ significantly (* $P < 0.05$) from the food intake value for that day.

BHT (500 mg/kg/day) declined during the 6 days of treatment (Table 4). This discrepancy may be the result of differences in food intakes of the animals in these two studies: the former authors noted that the rabbits failed to eat, whereas in the present study food intakes were diminished but not abolished (Table 1). Short-term starvation of rabbits has been shown to induce a natriuresis and diuresis in the initial 24 hr (Cizek, Simchon & Nocenti, 1977); an increase in water intake followed on the second day of fasting and appeared to be subsequent to reduction of plasma volume. The osmolality of the plasma from fasting rabbits was reduced, although no changes were observed in plasma sodium, potassium, and protein concentrations (Cizek *et al.* 1977; Nohno, Hayashi & Murayama, 1977).

The physiological basis of the natriuresis of fasting has not been determined with certainty. Microperfusion studies in rats fasted for 4–5 days indicated that urine flow and sodium excretion increased while the clearance of inulin and excretion of urea were depressed (Murayama, Watanabe & Hayashi, 1976). Aldosterone secretion was elevated in the fasting state, even when sodium and potassium were added to the drinking water to prevent electrolyte depletion (Nohno *et al.* 1977). This increase in mineralocorticoid production appears to be insufficient to prevent the sodium losses accompanying food deprivation, and may explain the observations of Denz & Llaurado (1957).

The electrolyte losses during fasting are dependent upon caloric deprivation, inasmuch as a salt-free diet has been shown to produce an adequate conservation of sodium (Cizek *et al.* 1977; Nohno *et al.* 1977). A role for the glucoregulatory hormones in electrolyte balance has been suggested. Increased plasma insulin concentration stimulates retention of both sodium and potassium by the kidneys whereas glucagon increases the urinary excretion of sodium, potassium, calcium, phosphorus and magnesium; this is apparently by direct action on the renal tubule (Knochel, 1977). Glucagon concentrations in the plasma are elevated during fasting (Marliss, Aoki, Unger, Soeldner & Cahill, 1970) and it is possible that the natriuresis and diuresis observed are mediated through the action of this hormone.

On the second day of BHT treatment in the present study electrolyte excretions were greater than expected from food intake (Fig. 1). The enhanced electrolyte excretion was accompanied by increased urine volume (Table 2). Inasmuch as food intake was concurrently depressed by 73% (Fig. 1), these observations might be explained by an increase in plasma glucagon. With increased food consumption on days 4 and 6, the excretion of electrolytes once again became proportional to intake. The diuresis which followed the fourth dose of BHT (Table 2) could have been maintained by polydipsia resulting from plasma volume depletion (Cizek *et al.* 1977).

During administration of BHA for 6 days, potassium excretion was concordant with food intake (Fig. 1). Daily sodium output, however, was significantly reduced following the second dose. Inasmuch as the water balance of these animals was not affected (Table 2) and the osmolality of the urine remained constant (Table 3) a defect in renal concentrating ability is unlikely. Renal prostaglandins (PG) modulate urine flow and sodium excretion in laboratory animals (Terragno, Malik, Nasjletti, Terragno & McGiff, 1976). BHA (1·0 mM) has been shown to decrease the production of PG_{E2} and PG_{F2a} by slices of renal medulla, while BHT had no effect (Zenser & Davis, 1978). Donaldson (1973) noted a decrease in activity of bradykinin (assayed with a uterine horn preparation). The apparent sodium retention observed with BHA treatment (Fig. 1) therefore might be caused by alterations in renal prostaglandin metabolism, either by interference with cyclo-oxygenase activity (Zenser & Davis, 1978) or through inactivation of bradykinin (Donaldson, 1973) which is thought to activate phosphorylase (Terragno *et al.* 1976). Alternatively, BHA could depress sodium excretion by affecting its absorption in the gut. BHA has been shown to depress the sodium-dependent processes of glucose and methionine absorption in rat intestinal preparations (Fritsch, Lamboeuf & de Saint Blanquat, 1975). Impaired sodium absorption might also be mediated through alterations in intestinal prostaglandin metabolism (Beubler & Juan, 1976).

In summary, electrolyte excretion is reduced in animals treated with 500 mg/kg/day BHA or BHT; this accompanies the reduction of food intake which occurs with large doses of these antioxidants. The disproportionate electrolyte excretions observed on the second day of BHT treatment may be related to the severe depression of food intake. The depression of sodium excretion noted with BHA administration may be due to impaired renal function or to decreased sodium absorption in the intestine.

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Fd Cosmet. Toxicol. Vol. 18, pp. 21 to 26

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THE EFFECTS OF BUTYLATED HYDROXYANISOLE AND BUTYLATED HYDROXYTOLUENE ON RENAL FUNCTION IN THE RAT. II. EFFECTS ON ORGANIC ACID AND BASE TRANSPORT

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Abstract—A previous study indicated that administration of the food additives butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) adversely affects electrolyte balance in rats. This paper describes experiments in which organic ion transport was used as another index of renal function. Male rats were treated with BHA or BHT (500 mg/kg) or corn oil for 1, 2, 4 or 6 days. Twenty-four hours after the last dose of antioxidant the animals were killed, and the ability of renal cortical slices to accumulate a prototype organic acid, *p*-aminohippurate and a prototype organic base, *N*-methylnicotinamide, was determined. Organic acid transport was reduced following the first dose of either antioxidant and continued to decrease with BHT treatment, reaching a minimum after four doses. After the sixth day of treatment however, *p*-aminohippurate accumulation was comparable among BHA- or BHT-treated groups and control animals. *N*-Methylnicotinamide transport was not affected at any time by treatment with either antioxidant. The results demonstrate that these phenolic antioxidants have a specific, depressive action on organic transport. The attenuation of this effect despite continual administration of antioxidant may be related to induction of hepatic metabolism.

INTRODUCTION

The effects of administration of the food additives butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on fluid and electrolyte excretion in the rat are reported in the preceding paper (Ford, Hook & Bond, 1980). In addition to maintaining electrolyte and water homeostasis, the kidneys are the major sites of excretion for many xenobiotics and metabolic by-products. Such substances may appear in the urine as the result of glomerular filtration and/or secretion in the proximal segment of the nephron. During the process of secretion in the intact animal, organic acids and bases are actively transported from the blood across the peritubular membrane, into the cells of the proximal tubule (Foulkes & Miller, 1959). Movement into the lumen is then accomplished by passive diffusion down a concentration gradient which is maintained by tubular flow.

BHA and BHT are metabolized in the liver to sulphate and glucuronic acid conjugates which are excreted in the urine (Hathway, 1966). These acidic derivatives may be secreted into the proximal tubule of the nephron by systems responsible for the transport of organic anions, in the same way as metabolites of other xenobiotics (Rennick, 1972). This active accumulation from the blood into the tubule, as well as the reabsorption of water along the nephron, would result in an increased concentration of BHA and BHT derivatives in the functional units of the kidneys.

The sequence of events during secretion in the proximal tubule is dependent upon patent biochemical and structural components and is sensitive to the effects of nephrotoxins in the blood (Foulkes & Hammond, 1975). Therefore, the secretory capacity of the kidneys has been used as an index of renal function in toxicological studies (Hirsch, 1976). Inhibition of secretory apparatus may be detected by depression of the transport capacity for a prototype ion, usually *p*-aminohippurate (PAH) for the organic acid system, and *N*-methylnicotinamide (NMN) for organic bases. Such inhibition is demonstrated *in vivo* by depression of the maximal transport capacity, T_m, using standard clearance techniques. However, interpretation of *in vivo* studies can be confounded by any humoral changes or alterations in renal blood flow which accompany treatment (Hirsch, 1976). In order to eliminate these factors, an *in vitro* technique described by Cross & Taggart (1950) is used to estimate secretory capacity. Renal cortical slices are incubated in a medium containing PAH and/or NMN. The ability of proximal tubular cells in the slices to accumulate these ions from the medium is expressed as a ratio of the concentration of the ion in the slices to the concentration in the medium (S:M ratio). The magnitude of the gradient established is an index of active transport across the peritubular membranes of the cells (Berndt, 1976a).

The effects of BHA or BHT treatment on renal tubular transport systems have not been examined, although a large proportion of the anionic metabolites of these compounds are excreted in the urine. The experiments described in this paper were undertaken to determine the effects of acute treatment with BHA or BHT on organic acid and base transport in

the kidney cortex, using the *in vitro* slice technique to estimate tubular secretion.

EXPERIMENTAL

Materials. The test compounds BHA and BHT were purchased from Sigma Chemical Co., St. Louis, MO. The PAH was obtained from Eastman Kodak Company, Rochester, NY and ^{14}C-NMN from New England Nuclear, Boston, MA. All other reagents were of standard laboratory grade.

Animals, diets, and treatments. Male Sprague-Dawley rats (Spartan Research Farms, Haslett, MI) weighing 275–299 g were individually housed and offered a grain diet (Schemmel, Mickelsen & Motawi, 1972) *ad lib.* Before initiation of treatment the animals were allowed to adjust to the cages for 3–4 days. BHA or BHT was dissolved in corn oil and administered by gavage at a dose of 500 mg/kg/day for 1, 2, 4 or 6 days. Control animals received a similar volume (5 ml/kg) of corn oil. At the end of the treatment periods the weights of the kidneys, livers and adrenal glands were noted.

Organic ion accumulation. Twenty-four hours following the last dose of antioxidant or corn oil, the animals were killed by cervical dislocation and the kidneys quickly removed and decapsulated. Thin slices of the renal cortex were prepared freehand and divided between duplicate beakers containing 2·7 ml of phosphate buffer medium at pH 7·4 (Cross & Taggart, 1950). The medium contained $7{\cdot}4 \times 10^{-5}$ M-PAH and $6{\cdot}9 \times 10^{-6}$ M-($2{\cdot}5 \times 10^{-2}$ μCi/ml)[^{14}C]-NMN. The samples were incubated at 25°C in a Dubnoff metabolic shaker at 100 rpm in an atmosphere of 100% oxygen.

After 90 min the slices were blotted, weighed, and homogenized in 5 ml 10% trichloroacetic acid (TCA). A 2-ml aliquot of the incubation medium from each sample was added to 3 ml 10% TCA. The volumes of the slice and medium preparations were brought to 10 ml with distilled water and the samples centrifuged at 1400 rpm for 10 min. One-ml aliquots of the supernatants were assayed for PAH by a colorimetric method (Smith, Finkelstein, Aliminosa, Crawford & Graber, 1945). [^{14}C]NMN concentrations in the supernatants were determined by liquid scintillation counting. The slice to medium ratios (S:M) of PAH and NMN were calculated as the concentration of the organic ion in the tissue, divided by the respective concentration in the medium.

To determine whether the unmetabolized form of BHA or BHT can inhibit PAH and NMN transport, both antioxidants were separately added to the medium in which slices from untreated animals were incubated. Because solubilizers such as DMSO were not found satisfactory for use in the slice preparation (unpublished observations) the antioxidants were dissolved in ethanol and added to incubation beakers. The ethanol was evaporated, leaving a film of BHA or BHT on the glass. The medium was added and slices incubated as before. The concentrations in the beakers would have been $1{\cdot}5 \times 10^{-1}$ M, $1{\cdot}5 \times 10^{-2}$ M, $1{\cdot}5 \times 10^{-3}$ M and $1{\cdot}5 \times 10^{-4}$ M had all of the antioxidant gone into solution. Ethanol alone was added to control beakers and allowed to evaporate.

The effects of reduced food intake were examined by determining organic acid transport after 1, 2 or 4 days of fasting, or after a regimen involving four days of fasting and two days of *ad lib.* feeding.

Statistics. Data were subjected to analyses of variance and differences among the means were tested with Duncan's multiple range test, using $P < 0{\cdot}05$ as the criterion of significance (Steel & Torrie, 1960).

RESULTS

Organic ion transport

The ability of renal cortical slices to accumulate PAH was depressed 24 hr after a single dose of BHA or BHT, compared with corn oil (Table 1). This depression was also evident in both treated groups after two doses of either antioxidant. Following four doses, the PAH S:M ratio for slices from BHA-treated rats had begun to approach control values and the S:M ratio from the BHT-treated animals reached a minimum. Despite continual administration of the antioxidants, the organic acid transport capacity of slices from animals treated with BHA or BHT did not differ significantly from controls after six doses.

Table 1. *Effect of administration of BHA or BHT (500 mg/kg/day) on accumulation of organic ions by renal cortical slices*

Treatment	Accumulation of organic ions by cortical slices (S:M ratio)†			
	Day of treatment ... 1	2	4	6
		PAH		
BHA	6·33 ± 0·44*	6·50 ± 0·47*	8·08 ± 0·24	7·48 ± 1·02
BHT	6·11 ± 0·95*	4·80 ± 0·51*	3·96 ± 0·78*	7·51 ± 1·23
Corn oil	8·94 ± 0·76	10·52 ± 0·70	10·19 ± 1·13	8·64 ± 0·70
		NMN		
BHA	7·39 ± 0·98	6·32 ± 0·52	6·26 ± 0·23	5·63 ± 0·24
BHT	5·67 ± 0·29	6·03 ± 0·37	6·16 ± 0·37	5·36 ± 0·27
Corn oil	6·15 ± 0·57	5·89 ± 0·22	5·94 ± 0·60	6·08 ± 0·45

BHA = Butylated hydroxyanisole BHT = Butylated hydroxytoluene
PAH = *p*-Aminohippurate NMN = *N*-Methylnicotinamide
†See text for explanation.
Values are means ± SEM for four experiments and those marked with asterisks differ significantly (Duncan's multiple range test) from the control value (*$P < 0{\cdot}05$).

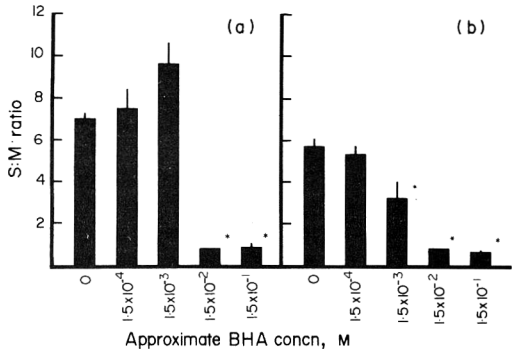

Fig. 1. Effect of BHA on organic acid (a; PAH) and base (b; NMN) accumulation by renal cortical slices. BHA was added to the medium in which slices from untreated rats were incubated. Each bar represents the mean ± SEM of four experiments; those marked with asterisks differ significantly (Duncan's multiple range test) from control values (*$P < 0.05$).

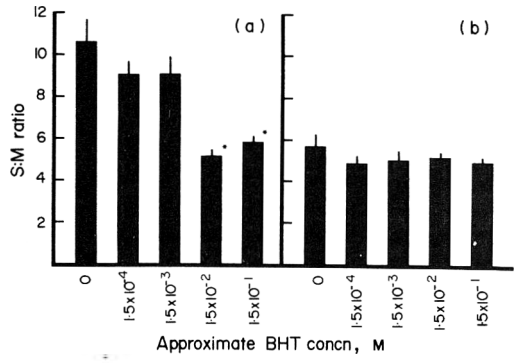

Fig. 2. Effect of BHT on organic acid (a; PAH) and base (b; NMN) accumulation by renal cortical slices. BHA was added to the medium in which slices from untreated rats were incubated. Each bar represents the mean ± SEM of four experiments; those marked with asterisks differ significantly (Duncan's multiple range test) from control values (*$P < 0.05$).

There were no significant effects of administration of either antioxidant on organic base transport as measured by NMN accumulation (Table 1).

Addition of BHA to the incubation medium of renal cortical slices from untreated rats depressed PAH and NMN accumulation at the two highest concentrations used (Fig. 1). The S:M ratio in both cases was less than 1·0. BHA resulted in decreased NMN S:M ratio at lower levels than those at which the PAH S:M ratio was affected.

BHT in the medium produced a significant depression of PAH transport at the two highest concentrations used (Fig. 2). NMN accumulation was not affected at any level of BHT.

Organ weights

The kidney weight of treated animals was not affected by administration of either antioxidant (Table 2). Increased adrenal gland weight was noted in animals receiving BHT; a maximum and significant increase occurred following the fourth dose (Table 2). No effect on adrenal weight was evident with BHA treatment.

Following the second dose, the liver from animals receiving BHA was significantly heavier than that of corn oil-treated rats. The liver weight of animals treated with BHA had returned to the control level after six doses. BHT treatment also resulted in increased liver weight; it was not significant until day 4, but reached a higher final value than with BHA.

Food restriction

The ability of cortical slices from fasting animals to accumulate PAH was depressed throughout the period of restriction (Table 3), attaining control levels only after resumption of feeding.

DISCUSSION

The present study was designed to examine the effects of BHA and BHT on renal organic ion secretion in rats at a dose (500 mg/kg/day) that is known to produce profound alterations in other organ systems (Allen & Engblom, 1972; Witschi & Côté, 1976), but without death. The secretion of organic ions by the proximal tubule was estimated *in vitro*

Table 2. *Organ weights of animals treated with BHA or BHT*

Treatment	Weight of organ			
	Day of treatment ... 1	2	4	6
		Kidneys (g)		
BHA	2·46 ± 0·13	2·32 ± 0·03	2·36 ± 0·09	2·31 ± 0·03
BHT	2·35 ± 0·05	2·45 ± 0·12	2·22 ± 0·05	2·23 ± 0·10
Corn oil	2·54 ± 0·06	2·39 ± 0·10	2·36 ± 0·07	2·46 ± 0·10
		Liver (g)		
BHA	13·67 ± 0·94	15·90 ± 0·60*	16·07 ± 1·44*	15·61 ± 0·35
BHT	12·77 ± 1·02	14·75 ± 0·57	16·17 ± 1·02*	18·35 ± 1·18*
Corn oil	13·27 ± 0·94	13·14 ± 1·04	13·52 ± 0·48	13·91 ± 0·63
		Adrenals (mg)		
BHA	47·4 ± 1·2	44·2 ± 3·3	49·7 ± 4·1	53·2 ± 4·3
BHT	55·1 ± 4·2	53·2 ± 3·8	61·1 ± 7·8*	54·1 ± 2·7
Corn oil	44·7 ± 2·1	46·2 ± 3·7	46·7 ± 3·0	49·9 ± 4·5

BHA = Butylated hydroxyanisole BHT = Butylated hydroxytoluene
Values are means ± SEM for groups of four animals and those marked with asterisks differ significantly (Duncan's multiple range test) from the control value (*$P < 0.05$).

Table 3. *Effect of food restriction on the accumulation of PAH by renal cortical slices*

Treatment	PAH S:M ratio†			
	Day of treatment . . . 1	2	4	6
Restricted feeding	6·53 ± 0·20*	6·97 ± 0·17*	7·70 ± 0·76	10·12 ± 1·41‡
Control (fed *ad lib.*)	9·30 ± 1·24	9·34 ± 0·69	9·32 ± 1·17	9·43 ± 0·98

PAH = *p*-Aminohippurate

†Ratio determined at the end of the designated day; see text for explanation.

‡Animals were fasted for 4 days, then returned to *ad lib.* feeding for 2 days.

Values are means ± SEM for four experiments and those marked with asterisks differ significantly (Duncan's multiple range test) from the control value (*$P < 0·05$).

in order to eliminate extra-renal factors. Although the normal morphology of the nephron is disrupted in the slice system, this method has been shown to have validity for comparison of the effects of toxic compounds on secretory processes (Berndt, 1976a). Because of its dissociation from haemodynamic and humoral influences, the slice technique is at present the most sensitive method available for the detection of toxic effects on organic anion and cation transport (Berndt, 1976b).

Fasting has been reported to depress PAH accumulation (Hook & Munro, 1968) and it is possible that the inhibitory effects of BHA and BHT may be due to some factor associated with decreased food intake. However, the reduction of the PAH S:M ratio noted even after four days of total food deprivation (Table 3) was not of sufficient magnitude to account for the results in Table 1.

The processes of organic ion secretion are dependent upon metabolic energy, and if the production or utilization of this energy were reduced, it would be expected that transport capacity be compromised. Organic base accumulation was not affected by either compound in this study (Table 1); therefore, a non-specific depression of renal metabolism is unlikely.

The accumulation of organic anions by renal cortical slices is an index of the transport capacity of the peritubular membrane (Berndt, 1976a). Membrane-bound transport mechanisms are sensitive to factors that alter the lipid and protein constituents of the phospholipid bilayer (Peters, 1977). BHA and BHT are lipid-soluble molecules, and as such are capable of associating with and altering the characteristics of liposomes (Singer & Wan, 1977; Zilber, Dubur, Kumsar & Velena, 1971). If a physicochemical interaction with the peritubular membrane did occur, effects on organic base transport would have been expected as well.

BHA and BHT have been found to bind to proteins and polypeptides such as albumin and bradykinin (Donaldson, 1973; Gilbert, Martin, Gangolli, Abraham & Golberg, 1969). Possibly they exert their effects on PAH transport by interacting with a specific protein involved in the process of accumulation of organic anions. Interference with intracellular binding sites for PAH, or a carrier located in the membrane, would depress the gradient of the ion established by the slices (Berndt, 1976a). The activity of such transport systems might also be diminished by inhibition of protein synthesis in the cells. Milner (1967) has demonstrated significant reductions of DNA, RNA and protein syntheses in cultured monkey kidney cells within 30 min of addition of 0·034–0·136 mM-BHT to the media. This inhibition was reversed when the media were replaced with media containing no BHT.

The effects of BHT treatment on laboratory animals are often more pronounced than those of BHA, as for example the increases in liver weight and induction of drug-metabolizing enzymes noted in several studies (Brown, Johnson & O'Halloran, 1959; Johnson & Hegwill, 1961; Martin & Gilbert, 1968). In several cases the effects of either antioxidant are diminished or remain constant in spite of continual administration (Feuer, Golberg & Le Pelley, 1965; Gilbert & Golberg, 1965). Similar features were found in the depression of organic anion accumulation by renal cortical slices from animals treated with BHA or BHT (Table 1). During the six days of treatment the PAH S:M ratio declined to a lower value following BHT than BHA, and the recovery was faster in BHA-treated groups, reaching control values on the fourth day. The accumulation of the organic anion by renal slices returned to control values by the sixth day despite continued treatment with either antioxidant.

These results may be related to the rate at which the compounds are metabolized. BHA is conjugated in the liver and the water-soluble metabolites are quickly excreted (Hathway, 1966). BHT, in contrast, undergoes a series of oxidations before being conjugated. This factor, in addition to enterohepatic circulation, results in delayed excretion of BHT. The protracted elimination is associated with the degree of liver enlargement and induction of mixed-function oxidases; presumably the persistence of BHT in the body is a continuous stimulus to liver growth (Gilbert *et al.* 1969; Schulte-Hermann, 1974). The ability of the animal to metabolize BHT may thus be increased and toxic manifestations of the parent compound diminished.

The results noted with addition of BHA or BHT to the incubation medium (Figs 1 & 2) support the suggestion that the unmetabolized form of these compounds is capable of depressing organic ion transport. The effect of BHT *in vitro* was specific for organic anion transport, as found *in vivo*.

Increases in the liver weight of the rats occurred following treatment with either antioxidant (Table 2). The liver weight of animals treated with BHA reached a plateau following two doses, subsequently the PAH S:M ratio of these rats began to approach control values (Table 1). If the agent responsible for the

depression of organic acid accumulation were the unmetabolized form, this observation suggests that after two doses the hepatic ability to metabolize BHA is adequate for the dose presented. The changes in liver weights following one to four doses of BHT were comparable to BHA (Table 2); however, PAH accumulation in the BHT-treated rats was depressed to a greater extent. The PAH S:M ratio returned to control values only after the liver weights of the BHT-treated animals were significantly greater than those of the animals treated with BHA. Again, if the presence of unchanged BHT were responsible for the observed effects on PAH accumulation, then it is likely that the hepatic capacity to metabolize 500 mg/kg/day BHT is not sufficient until the sixth day.

Increased adrenal weight has been noted in association with BHT. treatment (Gaunt, Feuer, Fairweather & Gilbert, 1965; Schoebesch, 1962). The design of the present experiments permitted study of the time-course of changes in adrenal weight. From Table 2 it appears that BHA had no significant effect on the weight of the adrenal glands, whereas a consistent (but not always significant) increase was noted with BHT treatment. The adrenals were 23% heavier following one dose and a maximum of 31% was reached on the fourth day of treatment. By the sixth day, however, no differences were apparent among the groups. Increased adrenal weight has been interpreted to indicate stress (Sporn & Schoebesch, 1961). It has also been suggested that this represents hyperfunctional enlargement due to the presence of drug-metabolizing enzymes in the adrenal glands (Gaunt *et al.* 1965). Inasmuch as aldosterone secretion is increased during the alterations of electrolyte balance accompanying antioxidant treatment (Denz & Llaurado, 1957), an alternative explanation might be offered by increased production of aldosterone.

The studies in this paper indicate that BHA and BHT have a depressant effect that is specific to organic anion transport. The diminution of PAH accumulation is more severe with BHT administration. The observation that PAH transport recovered by the sixth day despite continued antioxidant administration, suggests that the effect may be modified by changes in the metabolism of these compounds.

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Fd Cosmet. Toxicol. Vol. 18, pp. 27 to 29

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N-NITROSODIMETHYLAMINE IN BEER

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Abstract—Commercially-available bottled and canned beer was analysed for volatile nitrosamines using a chemiluminescence detector. *N*-Nitrosodimethylamine, the only volatile nitrosamine found, was detected in 23 of 25 samples analysed. The range of levels was 0–14 ppb (μg/kg) while the mean was 5·9 ppb. The identity of *N*-nitrosodimethylamine was confirmed by mass spectrometry.

INTRODUCTION

During the past decade there has been considerable interest in the analysis of foods for volatile *N*-nitrosamines. Although the primary focus has been on meat cured with nitrite (Scanlan, 1975), volatile nitrosamines have been shown to occur sporadically in other foods such as cheese and fish (Crosby, Foreman, Palframan & Sawyer, 1972; Eisenbrand, Speigelhalder, Janzowski, Kann & Preussmann, 1978; Gough, Webb & Coleman, 1978) and, most recently, in beer.

Speigelhalder, Eisenbrand & Preussmann (1979) analysed 158 samples of different types of beers commercially available in West Germany for volatile nitrosamines. Of these, 70% of the samples contained *N*-nitrosodimethylamine (NDMA), the mean concentration being 2·7 ppb (μg/kg). More recently Goff & Fine (1979) reported NDMA at levels ranging from 0·4 to 7·0 μg/litre in 18 brands of domestic (USA) and imported beer. In addition, six out of seven brands of Scotch whisky contained NDMA at levels between 0·3 and 2·0 μg/litre. The purpose of our work was to investigate further the occurrence of NDMA in beer produced in the United States.

EXPERIMENTAL

Canned or bottled beers produced in the United States were purchased at local retail outlets. Volatile nitrosamines were extracted from the samples by a distillation procedure similar to that described by Goodhead & Gough (1975). Into a 1 litre two-necked, round-bottomed boiling flask were introduced 300 ml beer, 180 g sodium chloride, 5 g ammonium sulphamate, 100 ml distilled water, and enough hydrochloric acid to adjust the pH to 1–2. The mixture was steam distilled until 600 ml of distillate had been collected. The distillate was saturated with sodium sulphate, adjusted to pH 2 with hydrochloric acid and extracted four times with 100 ml dichloromethane. The combined extracts were washed with 50 ml 6 M-hydrochloric acid which was back-washed with dichloromethane (2 × 30 ml). The pooled dichloromethane extracts were washed with 50 ml 5 M-sodium hydroxide, passed through a 15 mm OD column containing 30 g sodium bisulphite, and then passed through a funnel containing 100 g anhydrous sodium sulphate. The dried extract was collected in a Kuderna-Danish evaporator and concentrated to 4 ml at 60°C. The extract was further concentrated to 1 ml under a stream of nitrogen using a micro-Snyder column.

For detection and quantification, 4- to 8-μl aliquots of the concentrated extract were injected onto a 3·7 m long and 3·18 mm OD gas chromatographic (GC) column packed with 10% Carbowax 20 M on Chromosorb G-AW 60/120 mesh. The carrier gas was helium (flow rate: 25 ml/min), and the injector and column temperatures were 180°C. The column effluent was monitored using a chemiluminescence detector (Thermal Energy Analyzer, Thermo Electron Corp., Waltham, MA), the conditions for which were: furnace, 400°C; vacuum, 4 mm; trap −160°C.

Recovery was estimated by spiking beer that did not contain detectable levels of volatile nitrosamines with authentic compounds (3·3 ppb) and analysing samples as outlined above.

The detailed procedure used for mass-spectral confirmation of identity is described elsewhere (Hotchkiss, 1979). Essentially, a mini-volume valve (Carle Instrument Co., Fullerton, CA) installed between the end of the Carbowax 20 M packed column and the Thermal Energy Analyzer allowed diversion of the volatile nitrosamine for collection in a cooled piece of 25·4 cm long and 1·59 mm OD nickel tubing.

The trapped nitrosamine was later analysed by gas chromatography–mass spectrometry. The gas chromatographic (GC) inlet was fitted with a valving system that allowed trapped nitrosamines to be transferred directly onto the column. The GC conditions were: column, 180 m long and 0·5 mm ID Carbowax 20 M glass-support coated open tubular column; column temperature, 120°C; carrier gas, helium, 8 ml/min. The conditions for the Finnigan 1015C mass spectrometer were: filament current, 450 μA; electron voltage, 70 eV; analyser pressure, 10^{-6} Torr; data system, Systems Industries System 250 (Sunnyvale, CA).

RESULTS

The results from analysis of 25 samples of beer are summarized in Table 1. All but two samples con-

Table 1. *N-Nitrosodimethylamine content (ppb) of different types of beer produced in the USA*

Type of beer	No. of samples	NDMA content (ppb) Mean	Range
Pilsen lager	15	7·7	0–14
Low-calorie lager	3	4·0	0–7
Ale	2	5	3–7
Dark lager	2	0·7	0·5–0·8
Malt liquor	3	2·8	0·5–5

NDMA = *N*-Nitrosodimethylamine

The values are uncorrected for the level of recovery of NDMA, which was about 75%. The total of 25 samples represents 18 different brands, and the overall mean NDMA content for all samples is 5·9 ppb.

tained detectable levels of NDMA, the only volatile nitrosamine found in this study. The overall mean for all samples was 5·9 ppb NDMA and the range was 0–14 ppb. Values in Table 1 are uncorrected for recovery which was approximately 75% for NDMA. The chemiluminescence detection limit for the volatile nitrosamines was 0·1 ppb.

A low-resolution mass spectrum obtained from a beer containing 1 ppb NDMA is shown in Fig. 1a while Fig. 1b is the spectrum of the authentic compound obtained on our instrument. The mass-spectral data unequivocally establish the identity of NDMA in the sample of beer. Mass-spectral confirmation was obtained on eight of the beer samples listed in Table 1. The minimum level of NDMA necessary for mass-spectral confirmation was 1 ppb.

DISCUSSION

The results from our limited study generally agree with the surveys by Spiegelhalder *et al.* (1979) and Goff & Fine (1979); NDMA is the principle volatile nitrosamine in beer and it is present at levels of a few ppb.

The carcinogenic action of NDMA in experimental animals has been extensively investigated (Magee, Montesano & Preussmann, 1976). In spite of this, the minimum dose of NDMA in the human diet that poses a hazard is unknown. Among the cured meats, fried bacon has received considerable attention (*Federal Register* 1978, **43**, 20992) because it consistently contains trace levels of nitrosopyrrolidine (NPYR). It is interesting to compare the amounts of volatile nitrosamines ingested by consuming fried bacon and beer. For example, consumption of 25 g of fried bacon (approximately 4 oz or 113 g of bacon before frying) containing 10 ppb NPYR results in an intake of 0·25 μg of NPYR. By comparison 0·95 kg of beer (approximately 1 US quart or 0·95 litre) containing 5·9 ppb NDMA results in an intake of 5·6 μg of NDMA. In addition, animal feeding experiments indicate that, of the volatile nitrosamines, NDMA is one of the more potent carcinogens. Archer & Wishnok (1977) estimated that NDMA was six times more potent as a carcinogen than NPYR in the BD rat. Similarly Preussmann, Schmahl, Eisenbrand & Port (1977) estimated from dose-response feeding studies with rats that the no-effect level of NPYR was between two and a half and five times higher than that of NDMA. Although it is not known whether the amounts of NDMA ingested in beer are harmful, it would seem prudent to reduce the levels if possible.

Research to identify the source of NDMA in beer has been initiated, and studies to date indicate that malt produced by direct-fired kilning is the primary, if not the sole, source of NDMA. Studies designed to elucidate the mode of formation of NDMA and to explore methods to prevent it are currently underway.

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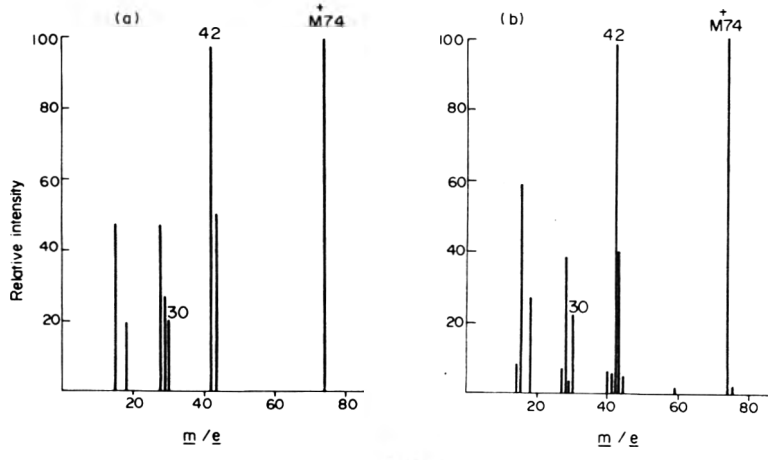

Fig. 1. Mass spectra of *N*-nitrosodimethylamine from (a) beer (b) authentic compound.

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N-NITROSAMINES—CONTAMINANTS IN BLOOD-COLLECTION TUBES

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Abstract—Volatile nitrosamines were found in commercially produced rubber-stoppered blood-collection tubes. Some rubber stoppers contained up to 147 ppb nitrosodimethylamine, 92 ppb nitrosodiethylamine and 1302 ppb nitrosomorpholine. These nitrosamines readily leached out of the stoppers to contaminate the tubes and their contents.

INTRODUCTION

Volatile nitrosamines, potent animal carcinogens, have been reported in human urine (Kakizoe, Wang, Eng, Furrer, Dion & Bruce, 1979), faeces (Wang, Kakizoe, Dion, Furrer, Varghese & Bruce, 1978) and blood (Fine, Ross, Rounbehler, Silvergleid & Song, 1977). During a recent study on nitrosamines in human blood (Lakritz, Simenhoff, Dunn & Fiddler, 1970), we observed unexpectedly high levels of *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA) and *N*-nitrosomorpholine (NMOR) in a blood sample. The latter compound has not previously been detected in significant quantities in biological samples or in foods. This report is the result of an investigation to determine whether the nitrosamines found were normally present in the blood or were artefacts introduced into the sample.

EXPERIMENTAL†

Materials. Dichloromethane (DCM), acetone and isooctane were obtained predistilled in glass from Burdick and Jackson (Muskegon, MI). Hydrochloric acid, sodium hydroxide and sodium sulphate (anhydrous) were ACS grade. All the reagents and distilled water used were checked and found to be free of nitrosamines.

Evacuated blood-collecting specimen tubes produced by four manufacturers (Kimble-Terumo, Elkton, MD; Jelco, Raritan, NJ; Becton-Dickinson & Co., Rutherford, NJ; Corning Glass Works, Corning, NY) were analysed for nitrosamines and amines. The tubes contained no additive and were silicone-coated or contained as an anticoagulant either sodium heparin, ethylenediaminetetraacetic acid (EDTA), sodium citrate, potassium oxalate or serum separator.

Methods. Rubber stoppers were removed from the blood-collection tubes and the exposed upper portion of each was removed to preclude the possibility of contamination from external sources. The lower portion of the stopper, which was in contact with the test tube, was immersed in 3 ml DCM for 30 min. The DCM was extracted three times with 3 ml 0·2 N-HCl, which in turn was re-extracted with an equal volume of DCM. The combined DCM extracts were dried by passage through anhydrous sodium sulphate and concentrated to 1·0 ml in a Kuderna-Danish apparatus on a steam bath and in a micro Snyder column in a water-bath at 68°C. The aqueous hydrochloric acid extracts, containing the free amines, were taken to dryness by lyophilization. The samples were reconstituted to 1·0 ml with water, and sufficient 0·2 N-NaOH solution was added to attain a pH of 10 prior to gas-chromatographic analysis.

The amines and nitrosamines inside the glass test tubes were removed by rinsing the tubes, including the additives, if any, with 3 × 3 ml 0·2 N-HCl and this acid solution was extracted with an equal quantity of DCM. The acid extract containing the amines and the DCM extract containing the nitrosamines were then concentrated as described above.

Analyses. The analysis for nitrosamines was accomplished by the use of a gas–liquid chromatograph (GLC) interfaced with a Thermal Energy Analyzer (TEA) Model 502 (Waltham, MA), which is extremely sensitive and specific for nitrosamines (Fine, Rufeh & Gunther, 1973). The nitrosamines were separated on a Varian-Aerograph Model 1720 gas chromatograph (Palo Alto, CA) containing a nickel column (9 ft × 1/8 in. OD) packed with 15% Carbowax 20 M-TPA on 60–80 mesh Gas Chrom P. The injector port temperature was 220°C, and the column temperature was programmed from 110 to 220°C at 4°C/min. With a helium flow rate of 42 ml/min, NDMA, NDEA and NMOR eluted at 4·2, 5·6 and 15·8 min, respectively. The TEA was operated under conditions similar to those reported by Fine & Rounbehler (1975). Samples containing concentrations as low as 0·1 ng/10 μl could be detected readily.

Apparent nitrosamines identified on the basis of GLC retention times and TEA detectability were confirmed by being exposed to ultraviolet light and then rechromatographed on the GLC–TEA (Doerr &

*Agricultural Research, Science and Education Administration, US Department of Agriculture. The products tested in this study were selected on the basis of availability. Reference to brand or firm name is given for the convenience of the reader and does not constitute endorsement by the US Department of Agriculture of any specific product over others.

†Note: Precaution should be exercised in the handling of nitrosamines since they are potential carcinogens.

Fiddler, 1977) to determine their photolability. Selected samples were further confirmed by separation and identification of the nitrosamines by high-pressure liquid chromatography (HPLC) interfaced with a TEA detector (HPLC–TEA). The nitrosamines were separated on a Laboratory Data Control HPLC equipped with a Constametric Pump II (Riviera Beach, FL) and a column (50 cm × 2·1 mm ID) packed with MicroPak Si 10 (10 μl) porous silica. The isocratic mobile phase was 5% acetone in isooctane operating at a flow rate of 1 ml/min. Elution times for NDEA, NDMA and NMOR were 6·3, 12·4 and 14·6 min respectively.

GLC-mass spectrometric analysis. For confirmation of the identity of the nitrosamines, a Varian-Aerograph Model 2700 GLC equipped with a glass column (6 ft × 1/4 in. OD) packed with 15% Carbowax 20M-TPA on Gas Chrom P was connected to a Varian MAT 311A mass spectrometer (MS; Varian Associates, Florham Park, NJ). The helium flow rate was 15 ml/min. The temperatures used for the detector, injector port and GLC–MS interface system were 200, 200 and 180°C, respectively, and the column was programmed from 90 to 140°C at 4°/min for NDMA and NDEA, and from 140 to 180°C at 6°/min for NMOR. The MS was operated in the peak-matching mode adjusted to a resolution of 1 in 10,000 or 12,000. The mass spectra were obtained at an ionizing voltage of 70 eV and an ion-source temperature of 150°C. The mass-to-charge ratios (*m/e*) of 74·04799 for NDMA and 102·07930 for NDEA were determined on the bases of the *m/e* 69·99857 and *m/e* 99·99361 perfluorokerosene reference peaks, respectively, by measuring the difference in *m/e*. The *m/e* of 116·05857 for NMOR was determined by using the *m/e* 106·07825 reference peak of xylene in a similar manner. The signal was recorded on both an oscilloscope and a recording oscillograph.

Confirmation of the nitrosamines by MS required larger samples than those used for TEA analysis. For each analysis, the lower portions of ten stoppers were ground and extracted twice with 20 ml DCM, and the extract was passed through an acid Celite column and concentrated as described above.

The amines were quantitated by GLC on a Hewlett-Packard Model 5710A gas chromatograph (King of Prussia, PA) equipped with a nitrogen–phosphorus detector. Separation was achieved in a glass column (6 ft × 2 mm ID) packed with 12% Amine 220 plus 8% KOH on 100–120 Chromosorb W/AW (Supelco, Bellefonte, PA). The injector, column and detector temperatures were 200, 50 and 250°C, respectively, for all separations except morpholine, for which the column temperature was 80°C. Carrier-gas flow rate was 15 ml/min at all times. At a column temperature of 50°C, the retention times for the amines were (in min): trimethylamine 1·2, methylamine 1·6, dimethylamine (DMA) 1·8, ethylamine 2·5 and diethylamine (DEA) 5·2. At 80°C, morpholine (MOR) eluted at 12·5 min.

RESULTS

No nitrosamines were detected in any of the reagents or distilled water used in the analytical procedures. Therefore, attention was directed to the blood-collection tubes and their anticoagulant contents (Table 1). Tubes manufactured by Kimble-Terumo had no detectable nitrosamines when empty, but those containing sodium citrate in solution showed nitrosamines up to 16 ppb NDMA, 9 ppb NDEA and 143 ppb NMOR. When a solution of EDTA was present as the anticoagulant, levels up to 17 ppb NDMA, 5 ppb NDEA and 158 ppb NMOR were found. Neither the Jelco brand blood-collection tubes, empty or containing solid heparin, nor the Becton-Dickinson and Corning brand tubes, empty or containing anticoagulants, contained detectable nitrosamines.

The presence of nitrosamines only in tubes with liquid anticoagulants suggested the possibility of the rubber stoppers as a source of the nitrosamines. Results of the analyses of rubber stoppers from major brands of evacuated blood-collection tubes are shown in Table 2. In 17 of 20 Kimble-Terumo tubes, rubber stoppers contained 11–98 ppb NDMA, 8–67 ppb NDEA and 85–678 ppb NMOR. The Jelco brand stoppers, although no nitrosamines were detected in the tubes, contained 39–147 ppb NDMA, 22–92 ppb NDEA and 359–1302 ppb NMOR. None of the Bec-

Table 1. *Nitrosamines in the contents of blood-collection tubes*

Manufacturer	Additive	Positive tubes (no./no. tested)	Nitrosamine levels (ppb)*		
			NDMA	NDEA	NMOR
Kimble-Terumo†	None	0/5	ND	ND	ND
	Na citrate (liquid)	4/5	5–16 (8)	3–9 (4·4)	61–143 (93)
	EDTA (liquid)	2/6	6–17 (5·5)	4–5 (1·5)	158‡ (26)
Jelco†	None	0/5	ND	ND	ND
	Heparin (solid)	0/6	ND	ND	ND
Becton-Dickinson†	None	0/3	ND	ND	ND
	Heparin (solid)	0/2	ND	ND	ND
	EDTA (liquid)	0/3	ND	ND	ND
	Na citrate (liquid)	0/2	ND	ND	ND

NDMA = *N*-Nitrosodimethylamine NDEA = *N*-Nitrosodiethylamine
NMOR = *N*-Nitrosomorpholine ND = None detected

*Ranges include positive samples only; values in brackets are the means.
†See footnote on p. 31.
‡Only one of the six samples was positive.

Table 2. *Nitrosamines in commercial rubber stoppers from evacuated blood-collection tubes*

Manufacturer	Additive	Positive stoppers (no./no. tubes tested)	Nitrosamine levels (ppb)* NDMA	NDEA	NMOR
Kimble-Terumo†	None	5/6	11–35 (21)	8–25 (16)	85–483 (266)
	Na citrate (liquid)	8/8	14–98 (66)	14–67 (41)	102–678 (420)
	EDTA (liquid)	4/6	20–91 (40)	15–66 (27)	144–467 (320)
Jelco†	None	7/7	39–117 (68)	42–92 (53)	359–1302 (770)
	Heparin (solid)	7/7	40–147 (69)	22–72 (41)	372–770 (514)
Becton-Dickinson†	None	0/3	ND	ND	ND
	Heparin (solid)	0/4	ND	ND	ND
	EDTA (liquid)	0/3	ND	ND	ND
	Na citrate (liquid)	0/4	ND	ND	ND
	K oxalate (solid)	0/3	ND	ND	ND
	Na fluoride (solid)	0/4	ND	ND	ND
Corning†	Serum-integrated (solid)	0/3	ND	ND	ND

NDMA = *N*-Nitrosodimethylamine NDEA = *N*-Nitrosodiethylamine
NMOR = *N*-Nitrosomorpholine ND = None detected

*Ranges include positive samples only; values in brackets are the means.
†See footnote on p. 31.

ton-Dickinson or Corning stoppers contained detectable concentrations of nitrosamines.

GLC-high resolution MS confirmed the presence of NDMA, NDEA and NMOR in the tested rubber stoppers. Analyses were carried out on selected lots of specimen tubes that indicated the possibility that they may contain large quantities of nitrosamines.

Because secondary amines are the most common precursors of nitrosamines, analyses for DMA, DEA and MOR were carried out. The amine and corresponding nitrosamine concentrations in a number of stoppers and collection-tube contents are shown in Table 3. Stoppers in the Kimble-Terumo and Jelco products contained substantial concentrations of the respective amines, ranging up to 1439 ppm MOR. The solution of sodium citrate in the Kimble-Terumo tube had low levels of DMA and DEA, but 99 ppm MOR. The liquid EDTA anticoagulant had 23 ppm MOR and less than 1 ppm of the other amines. Not more than 2 ppm of the amines was present in all other tubes analysed. Rubbers stoppers in which appreciable levels of amines were found invariably also contained nitrosamines.

Rubber septa used in GLC injection ports were examined to determine whether they too might contain nitrosamines, even though the possibility of sample contamination from this source seemed slight. Precut septa and rubber sheeting used to make septa were extracted for nitrosamines, and of five samples tested, one contained 36 ppb NMOR.

Table 3. *Concentration of amines and corresponding nitrosamines in stoppers and tubes*

Manufacturer	Additive	Sample	DMA (ppm)	NDMA (ppb)	DEA (ppm)	NDEA (ppb)	MOR (ppm)	NMOR (ppb)
Kimble-Terumo*	None	Stopper	72	10	69	8	707	85
		Contents	2	ND	2	ND	3	ND
	Na citrate (liquid)	Stopper	44	82	82	16	1081	569
		Contents	4	13	6	7	99	118
	EDTA (liquid)	Stopper	55	32	65	20	558	251
		Contents	<1	14	<1	5	23	79
Jelco*	None	Stopper	81	115	80	78	801	1015
		Contents	<1	ND	<1	ND	<1	ND
	Heparin (solid)	Stopper	67	102	78	55	1439	589
		Contents	<1	ND	<1	ND	2	ND
Becton-Dickinson*	None	Stopper	<1	ND	<1	ND	<1	ND
		Contents	<1	ND	<1	ND	<1	ND
	EDTA (liquid)	Stopper	<1	ND	<1	ND	<1	ND
		Contents	<1	ND	<1	ND	<1	ND
	Na citrate (liquid)	Stopper	<1	ND	<1	ND	<1	ND
		Contents	<1	ND	<1	ND	<1	ND

DMA = Dimethylamine NDMA = *N*-Nitrosodimethylamine DEA = Diethylamine NDEA = *N*-Nitrosodiethylamine
MOR = Morpholine NMOR = *N*-Nitrosomorpholine ND = None detected

*See footnote on p. 31.

DISCUSSION

Some amines and nitrosamines themselves are used in the formulation and curing of rubber. Aniline, substituted anilines and diphenylnitrosamines are used as antioxidants, and dimethylamine, *N*-(2,6-dimethylmorpholine)-2-benzothiazole and *p*-nitrosodiphenylamine are used as accelerators in the vulcanization of rubber (Kirk & Othmer, 1953). These nitrogenous compounds may be contaminated with nitrosamines, or they may react with nitrosating agents to form additional nitrosamines.

The data gathered in this study indicate that some, but not all, rubber stoppers obtained from blood-collection tubes contain nitrosamines, and that these compounds are readily leached into the liquids with which they come into contact.

The purpose of this communication is to alert other investigators to the possibility of accidentally introducing nitrosamine contaminants into their studies via products made of rubber. It is essential, therefore, that all blood-collection tubes be surveyed for these contaminants prior to use in studies on nitrosamines.

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THE 'CARRY OVER' OF AFLATOXIN M_1 INTO THE MILK OF COWS FED RATIONS CONTAINING A LOW CONCENTRATION OF AFLATOXIN B_1

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Abstract—Six dairy cows consuming a diet contaminated with approximately 10 μg aflatoxin B_1/kg excreted aflatoxin M_1 in the milk, the concentration varying between 0·01 and 0·33 μg/litre with a mean value of 0·19 μg/litre. Approximately 2·2% of ingested aflatoxin B_1 appeared in the milk daily as the metabolite aflatoxin M_1. This was a more consistent index of aflatoxin 'carry over' than the ratio of toxin concentrations in feed and milk.

INTRODUCTION

It is now well established that dairy cows consuming rations contaminated with aflatoxin B_1 excrete the toxic metabolite aflatoxin M_1 in the milk in dose-related concentration (see reviews: Kiermeier, 1977; Patterson, 1977). The literature of experimental 'carry over' published up to 1976 has also been reviewed by Rodricks & Stoloff (1977), who calculated from a compilation of published data that the ratio of the concentration of aflatoxin B_1 in the feed to that of aflatoxin M_1 in milk was very approximately 300:1.

In several surveys of liquid and dried milk carried out in recent years (Table 1), it has been amply confirmed that cows' milk often contains trace amounts of aflatoxin M_1, with peak concentrations occurring in the winter when the level of concentrate feeding is highest. Groundnut meal, cottonseed meal and maize are the ingredients of proprietary dairy concentrates or home-mixed rations most likely to contain aflatoxin. However, in the UK and other EEC countries their use is restricted by statute in such a way that the maximum levels of aflatoxin B_1 in concentrates and whole dairy rations are 20 and 10 μg/kg respectively (Fertilisers and Feeding Stuffs (Amendment) Regulations; Statutory Instrument 1976 No. 840).

Little detailed information has been published on the 'carry over' of aflatoxin at these very low dietary levels and the present communication, concerned with observations on our laboratory dairy herd, seeks to provide some relevant quantitative data.

EXPERIMENTAL

Milk samples were obtained from a small herd of Friesian and Friesian X dairy cows located at one of the laboratory farms. Their daily ration comprised hay (5 kg), a barley–protein mix (2 kg), and, depending on milk yield, 9–15 kg of a proprietary dairy concentrate.

All feedstuffs were analysed for aflatoxin B_1 by the standard thin-layer chromatographic method used throughout the EEC (UK SI 1976 No. 840, p. 60). Analysis of milk for aflatoxin M_1 was carried out using the method of Stubblefield (1979), recently tested in an international collaborative trial. Essentially the method involves extraction with chloroform in the presence of a strong solution of NaCl, and

Table 1. *Selected surveys of cows' milk for aflatoxin M_1, carried out in EEC countries*

Country	Total samples analysed	No containing aflatoxin M_1	Range of concentrations (μg/litre)	Reference*
Belgium	68	42	0·02–0·2	(1)
W. Germany	61	28	0·01–0·25	(2)
	419	79	trace–0·54	(3)
	260	118†	0·05–0·33	(4)
Netherlands	95	74	0·09–0·5	(5)
UK	278	85†	0·03–0·52‡	(6)

*(1) Van Pée, Van Brabant & Joostens, 1977; (2) Kiermeier, 1973; (3) Kiermeier, Weiss, Behringer, Miller & Ranfft, 1977; (4) Polzhofer, 1977; (5) Schuller, Verhülsdonk & Paulsch; (6) D. S. P. Patterson, unpublished data 1977.

†Seasonal effect, higher concentrations in winter corresponding with higher levels of concentrate feeding.

‡But 92·5% of the samples contained no more than 0·1 μg aflatoxin M_1/litre.

Table 2. *Aflatoxin B_1 content of dairy feedstuffs*

Feedstuff constituent	Aflatoxin B_1 (μg/kg)
Hay	Not analysed*
Barley, crushed	None detected
Protein balancer	40
Barley–balancer mix	<10†
Dairy nuts	15
Total feed‡	10·2 ± 0·68§

*No samples of hay analysed previously had been found to contain aflatoxin B_1.
†In calculating daily intakes of aflatoxin B_1, it was assumed that its concentration in the barley mix was 10 μg/kg.
‡Hay plus barley–balancer mix plus dairy nuts.
§Mean ± 1 SD; concentrations were calculated for the individual feed allowances of six cows.

purification on a silica-gel column, followed by quantitative thin-layer chromatography (TLC). Recoveries of aflatoxin M_1 added at a concentration of 0·5 μg/litre are about 80% (Stubblefield, 1979) and the detection limit is about 0·01 μg/litre.

In the use of either method, concentrations were determined in duplicate by comparing visually the fluorescence under ultraviolet light of unknown and standard amounts of aflatoxin B_1 or M_1 on a TLC plate. As noted elsewhere in relation to aflatoxin B_1 analysis (SI 1976 No. 840), the reproducibility of results between two or more laboratories would be of the order ± 50% of the mean values for concentrations in the range 10–20 μg/kg.

RESULTS

Samples of hay, home-grown barley, the proprietary balancer used to prepare the barley–protein mix, and the dairy nuts were ground mechanically and analysed in duplicate for aflatoxin B_1 content (Table 2). As the approximate feed intakes were also known, it could be calculated that individual cows ingested between 155 and 244 μg of the toxin daily.

In samples of bulked milk obtained from the herd at each morning milking for 7 days, the concentration of aflatoxin M_1 was found to vary from 0·15 to 0·26 μg/litre with a mean value of 0·21 μg aflatoxin M_1/litre. An identical mean value was obtained when milk samples from six cows were analysed at one milking but the concentration of aflatoxin M_1 was more variable, ranging from less than 0·01 to 0·33 μg/litre. Two of these cows, A and B, excreting aflatoxin M_1 at the lowest and highest levels at that time, were subsequently sampled at morning and afternoon milkings for five consecutive days. For cow A, the concentration of aflatoxin M_1 in morning milk ranged from 0·01 to 0·25 μg/litre and that in afternoon milk from 0·11 to 0·25 μg/litre. Corresponding ranges of values for cow B were 0·11–0·28 μg/litre for either milking.

The mean value for the daily quantity of aflatoxin M_1 excreted in the milk by six cows, based on analyses at one milking, was 4·28 μg per cow and the means for cows A and B, measured over a period of 5 days (ten milkings) were 3·27 and 3·48 μg, respectively (Table 3). Expressed as percentages of ingested aflatoxin B_1, these mean values for the daily output of aflatoxin M_1 were 2·64, 1·89 and 2·24%, respectively (Table 4).

Ratios of the feed concentrations of aflatoxin B_1 to milk concentrations of aflatoxin M_1 varied widely. Based on analytical data for the total feed, values ranged from about 29 to 989 and those based on the analyses of dairy nuts alone, from about 46 to 1500. The mean values for six cows at one milking were 201·3 (total feed) and 302·2 (dairy nuts only), values that were of the same order as the value derived by Rodricks & Stoloff (1977), but the coefficients of variation were approximately 192% in either case!

DISCUSSION

In the present study, the level of dietary aflatoxin contamination of about 10 μg aflatoxin B_1/kg total feed was the maximum permitted by the UK Fertilisers and Feeding Stuffs Regulations (SI 1976 No. 840), and using a recently developed sensitive analytical method, aflatoxin M_1 was detected in all but one of the milk samples examined. However, as found by Kiermeier (1977), concentrations of aflatoxin M_1 in milk appeared to vary from animal to animal, from day to day, and from one milking to the next, but the reproducibility of the analytical results was such (see

Table 3. *'Carry over' of aflatoxin M_1 into cows' milk from dairy rations containing 10·2 μg aflatoxin B_1/kg*

Parameter	Bulk milk*	Group of six cows†	Cow A‡	Cow B‡
Estimated total daily intake of aflatoxin B_1 (μg)	—	193·7 ± 41·2	173	155
Aflatoxin M_1 concentration (μg/litre milk)	0·21 ± 0·045§	0·21 ± 0·119	0·16 ± 0·073	0·19 ± 0·070
Milk yield (litres/day)	—	22·58 ± 4·03	20·6 ± 0·418	18·3 ± 0·837
Daily output of aflatoxin M_1 in milk (μg)	—	4·82 ± 2·38	3·27 ± 1·48	3·48 ± 1·45

*One milking on each of 7 days (from the whole herd).
†Samples taken at one milking only.
‡Two milkings/day for 5 days.
§Mean of five determinations at one milking only = 0·20 ± 0·029.
Values are means ± 1 SD for the numbers of determinations indicated.

Table 4. *A comparison of two indices of aflatoxin (AF) 'carry over' into cows' milk*

Origin of data	Daily AF M_1 output (% of estimated AF B_1 intake)		Ratio of AF concns			
			B_1 in whole feed:M_1 in milk		B_1 in dairy nuts:M_1 in milk	
	Mean ± SD	CV(%)	Ratio	CV(%)	Ratio	CV(%)
Six cows	2·64 ± 1·56	59·1	201·3 ± 386	191·8	302·6 ± 587	194·0
Cow A	1·89 ± 0·86	45·2	154·7 ± 294	190	234·6 ± 446	190·1
Cow B	2·24 ± 0·94	41·8	57·7 ± 20·9	36·3	91·1 ± 33·0	36·2

Values are means ± 1 SD. followed by the coefficient of variation (CV). Daily outputs and intakes were estimated for six cows at one milking only or for cows A and B on 5 days, combining data for two milkings per day. Ratios of AF concentrations in feed and milk were calculated for individual milk analyses, i.e. six cows at one milking or cows A and B at ten milkings.

Experimental section) that it was impossible to draw detailed conclusions on this aspect of the present data.

The highest concentration of aflatoxin M_1 measured in milk during the present experiment was 0·33 μg/litre, which is within the range of values found in our own and several published milk surveys (Table 1) and is 15 to 60 times lower than statutory limits in several countries for the maximum permitted concentration of aflatoxin B_1 in groundnuts and groundnut products used in the manufacture of human food (see reviews: Krogh, 1977; Stoloff, 1977).

Rodricks & Stoloff (1977) derived, from various pre-1976 sources, an approximate value of 300 for the ratio of aflatoxin B_1 concentration in feed to aflatoxin M_1 concentration in cows' milk, but reference to the individual publications they cited showed that this ratio varied considerably from animal to animal and from one set of data to another. Indeed, the overall range of values was 34 to 1600, which is similar to that obtained in the present study.

Whether calculated from data obtained from a group of six cows or from two individual cows, the output of aflatoxin M_1 expressed as a fraction of the calculated daily intake of aflatoxin B_1 was about 2·2% and compared well with previously obtained experimental data (0·35 to 3%; see review by Kiermeier, 1977). This parameter also appeared to be a more consistent index of 'carry over' than the ratio of toxin concentrations in feed and milk (Table 4) and might be useful in the prediction of aflatoxin M_1 concentrations in milk, provided the dietary concentration of aflatoxin B_1, feed intake data and a cow's daily milk yield are known. A regression formula previously deduced (Patterson, 1977) from published experimental data similar to that used by Rodricks & Stoloff (1977) seriously underestimated aflatoxin M_1 concentrations in milk at these low levels of dietary aflatoxin B_1 contamination.

Applying the analytical method for aflatoxin M_1 in milk, neither aflatoxin B_1 nor M_1 could be detected in plasma samples obtained from any of our six cows shortly after milking. As the minimum detection limit was 0·01 μg/litre and the mean concentration of aflatoxin M_1 in milk was some 20 times higher, it appears that toxin is excreted by the mammary gland against a concentration gradient and thus, probably, involves an active metabolic process. This fundamental aspect of aflatoxin 'carry over' and the public health significance of these very low concentrations of aflatoxin M_1 and possibly other metabolites in the milk are questions that remain to be resolved.

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Fd Cosmet. Toxicol. Vol. 18, pp. 39 to 45

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INFLUENCE OF CAPROLACTAM ON RAT-LIVER TYROSINE AMINOTRANSFERASE AND TRYPTOPHAN OXYGENASE

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Abstract—Caprolactam (2-oxohexamethylenimine), the monomer used in the production of Nylon 6, was tested for its ability to induce tyrosine aminotransferase (TAT) and tryptophan oxygenase (TPO) in rats. When a single oral dose of 1·5 g caprolactam/kg was given, maximum induction occurred in the livers after 6 hr and there were marked increases in enzyme activities after 3 and 24 hr. The induction was dose dependent, with a no-effect level for TAT of 300 mg/kg and for TPO of less than 300 mg/kg. In a paired-feeding study, it was shown that caprolactam inhibited the adaptive changes in liver-protein synthesis that were associated with a decrease in food intake. The induction of TAT by caprolactam did not require endogenous steroid, as increased enzyme activity was observed in adrenalectomized rats. Furthermore, the caprolactam induction did not appear to require RNA synthesis, since the induction was only partially blocked by inhibitors of RNA synthesis, actinomycin D or aflatoxin B_1. It is suggested that caprolactam induces changes in amino acid metabolism that bring about alterations in the activities of these enzymes.

INTRODUCTION

Caprolactam (2-oxohexamethylenimine) is the monomer used in the production of Nylon 6, and the annual production of this monomer in the United States is in excess of 500,000 tons. The oral LD_{50} of caprolactam in rats has been reported to be 2–4 g/kg (Lomanova & Preobrazhenskaya, 1961). In Fischer 344 rats, under the conditions of the studies reported here, the LD_{50} was approximately 2·5 g/kg (A. J. Salerno, unpublished data 1978). The acute lethal effects of caprolactam are rapid and appear to be mediated through direct cardiac and pulmonary toxicity. Caprolactam was also irritating to the rabbit eye (Smyth, Carpenter, Weil, Pozzani, Striegel & Nycum, 1969). Effects of caprolactam on reproductive physiology in rats and humans have been reported (Khadzhieva, 1969a,b), although other studies (McPhate & Fishbein, unpublished results 1962) have not confirmed these findings. In the published studies, levels of caprolactam exposure were not well quantified, and the contribution of other industrial chemicals to the observed effects was not ruled out. The limiting chronic toxicity of caprolactam in Sprague-Dawley rats appears to be renal toxicity, although this observation has not been confirmed (personal communication to Allied Chemical Corp., 1979).

Although no detailed metabolic studies on caprolactam have been performed, hydrolysis of the amide linkage would be expected to produce high levels of ϵ-aminocaproic acid. This substance might be expected to modify normal metabolic pathways in the body, as increases in non-essential amino acids or amino compounds have been shown to change anabolic and catabolic pathways. More specifically, ϵ-aminocaproic acid, being an amino acid, might also modify metabolic pathways involved in nitrogen metabolism (Munro, 1964). It was the purpose of the present studies to examine the influence of caprolactam on the metabolism of the amino acids tyrosine and tryptophan. Tyrosine aminotransferase (TAT) and tryptophan oxygenase (TPO) are enzymes involved in the first steps of the metabolism of tyrosine and tryptophan, respectively. These enzymes are under transcriptional and translational control, so that changes in dietary substrates or constituents increase or decrease their activities (Knox, 1966; Lin & Knox, 1957 & 1958). They are transcriptionally controlled by hormones such as glucocorticoids, insulin and glucagon; increases in glucocorticoid or glucagon levels increase their activity, but high levels of insulin suppress them. Actinomycin D and aflatoxin B_1, inhibitors of RNA synthesis, suppress hormonal induction (Lin & Knox, 1958; Wogan & Friedman, 1968). In contrast, exposure to aromatic amino acids causes a change in the enzyme proteins, stabilizing them against catabolism and thereby causing a net increase in enzyme activity (Knox, 1964; Schimke, Sweeney & Berlin, 1963). This type of induction is resistant to actinomycin D but sensitive to inhibitors of protein synthesis (Lin & Knox, 1958). Glucose-6-phosphatase and fructose-1,6-diphosphatase are enzymes involved in gluconeogenesis. Although the activity of these enzymes can change with the nutritional status of the animal, they do not affect amino acid metabolism. Therefore, they would appear to be useful comparisons for studies on amino acid metabolism.

EXPERIMENTAL

Animals. Male Fischer 344 rats, weighing between 80 and 100 g, were obtained from Charles River Breeding Laboratories (Wilmington, MA). They were housed in shoebox-type cages with constant access to Purina rat chow and water. In some cases, rats were

bilaterally-adrenalectomized at least 7 days before use and were maintained on isotonic saline instead of water.

Chemicals. Commercial-grade caprolactam was supplied by the Allied Chemical Corp., Fibers Division (Hopewell, VA). For animal treatment it was either dissolved in distilled water or mixed directly with ground Purina rat chow. Solutions of caprolactam were prepared immediately before use. Hydrocortisone acetate (150 mg/kg, ip) and tryptophan (600 mg/kg, ip) were used as positive controls and suspended in distilled water. Actinomycin D (1 mg/kg, ip) and aflatoxin B_1 (3 mg/kg in DMSO, ip) were used as inhibitors. All of these were obtained from Sigma Chemical Corp., St. Louis, MO. The times of injection and variations in doses are described in the experimental protocols, and appropriate solvent controls were always used.

Enzyme assays. Rats were killed by decapitation and exsanguination. The livers were removed, rinsed and homogenized in 0·14 M-KCl. The homogenates were frozen at −80°C and assayed within 2 wk. TAT activity was determined by measurement of the production of *p*-hydroxyphenylpyruvic acid from tyrosine (Friedman & Wrenn, 1977). TPO activity was determined by measuring conversion of tryptophan to kynurenine (Friedman & Wrenn, 1977). Glucose-6-phosphatase and fructose-1,6-diphosphatase activities were assayed by measuring production of inorganic phosphate from the substrate (Wogan & Friedman, 1968). Cytochrome *P*-450 was measured as described by Tucker, Tang & Friedman (1978) and liver-protein levels were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), but as there was no consistent change in liver-protein content, specific enzyme activities were not used. Enzyme activities are expressed as μmol product formed/g liver/hr. Data are presented as means ± SEM. For statistical analysis a variety of techniques was used (Snedecor & Cochran, 1968) depending on the original protocol.

Liver-protein synthesis. Liver-protein synthesis was quantitated in one study, 30 min after ip injection of [^{3}H]leucine (New England Nuclear Corp., Boston, MA). Samples of homogenate were treated with 5% TCA at 90°C for 15 min. Following centrifugation, the precipitate was washed twice with 5% TCA, once with ethanol, twice with ethanol–ether (3:1 v/v), and twice with ether. Following drying, samples of protein were weighed and oxidized in a Packard Oxidizer Model 306, and radioactivity was determined using a Beckman LS-9000 scintillation counter.

RESULTS

Time course of induction

Enzyme activities 3, 6 and 24 hr after oral administration of 1·5 g caprolactam/kg are shown in Fig. 1. Caprolactam increased the activity of both TPO and TAT and the maximum increase was observed 6 hr after treatment. At this time TAT and TPO activities were 964 and 723% of the control. After 24 hr, the level of induction was still significant: TAT activity was 443% and TPO activity 268% of the control. In contrast, glucose-6-phosphatase activity decreased after caprolactam treatment; 3 hr after treatment, glucose-6-phosphatase activity had decreased to 58% of the control value, and it remained low throughout the 24 hr after treatment. No significant change was observed in the activity of fructose-1,6-diphosphatase.

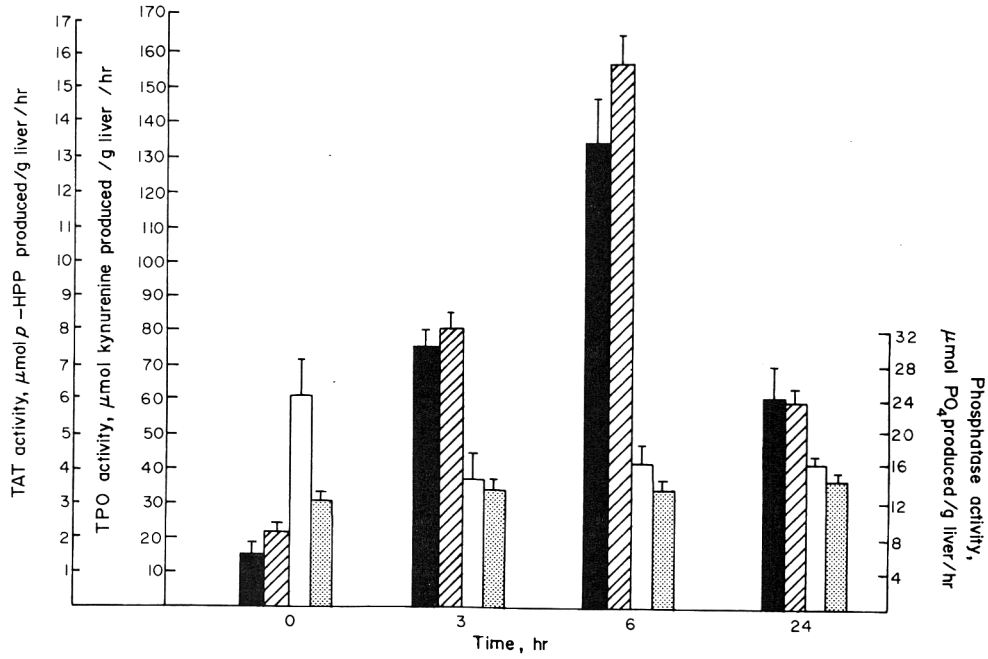

Fig. 1. Activities of tyrosine aminotransferase (TAT; ■), tryptophan oxygenase (TPO; ▨), glucose-6-phosphatase (□) and fructose-1,6-diphosphatase (▨) in liver homogenates from rats treated orally with 1·5 g caprolactam/kg and killed 3, 6 or 24 hr later. Values are means for groups of five rats and vertical bars indicate the SEM. Activities identified as 0 hr are the pretreatment controls.

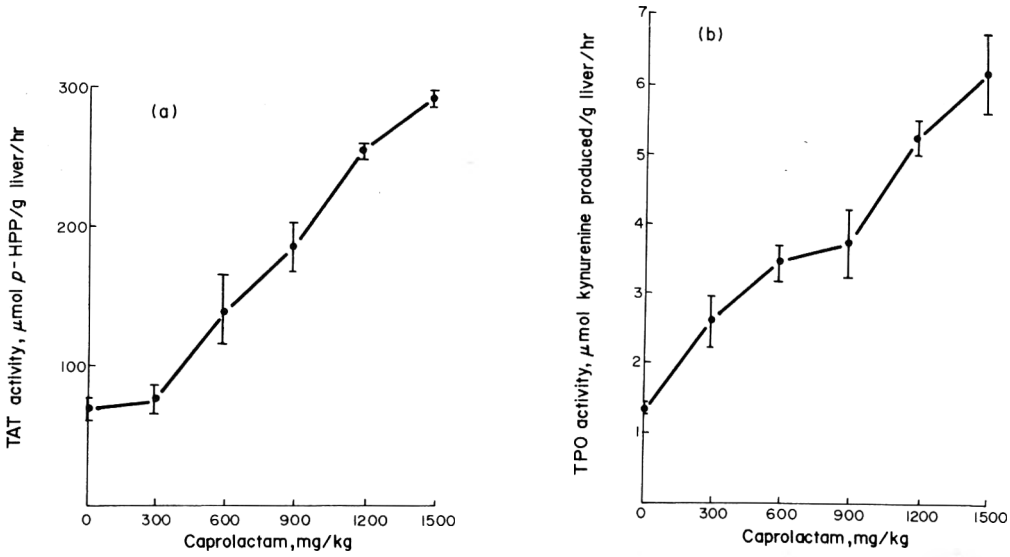

Fig. 2. Activities of (a) tyrosine aminotransferase and (b) tryptophan oxygenase in liver homogenates of rats given different oral doses of caprolactam and killed 5 hr later. Values are means for groups of five rats and vertical bars indicate the SEM.

Dose-response

The effects of various doses of caprolactam on TAT and TPO activity are shown in Fig. 2. There was no induction of TAT at 300 mg/kg (Fig. 2a), but enzyme activity increased by 100% at 600 mg/kg and continued to increase linearly with dose up to 1500 mg/kg, the highest dose tested. In the case of TPO (Fig. 2b), a 100% increase in enzyme activity was observed at 300 mg/kg and the enzyme activity continued to increase with dose up to 1500 mg/kg.

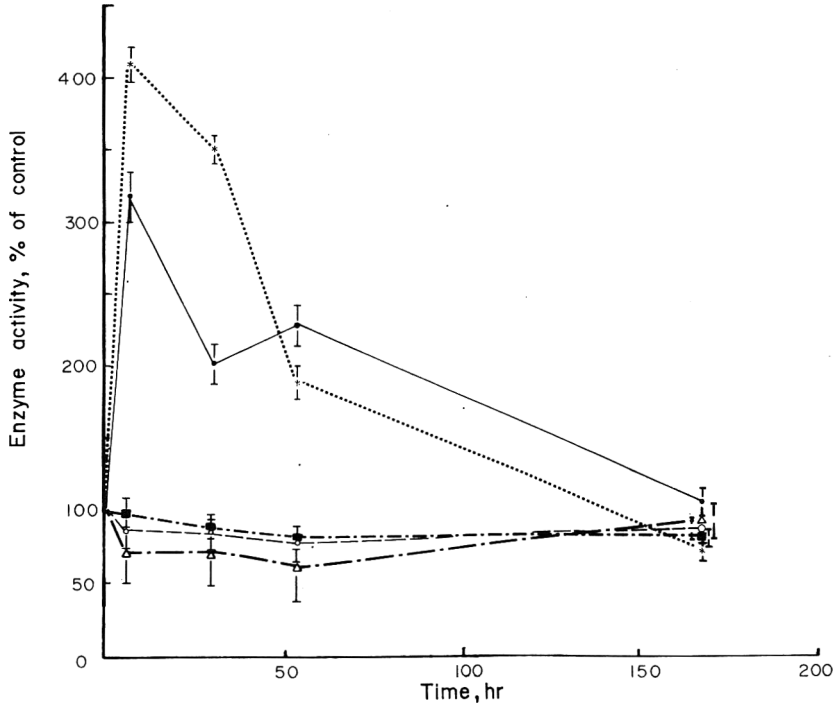

Fig. 3. Hepatic enzyme levels after daily ip injections of 500 mg caprolactam/kg. Rats were killed at the times indicated and tyrosine aminotransferase (TAT; —●—), tryptophan oxygenase (TPO; --*--), glucose-6-phosphatase (--○--), and fructose-1,6-diphosphatase (—■—) activities were determined and the cytochrome *P*-450 (—△—) level was quantitated. Enzyme activities and cytochrome *P*-450 levels are means for groups of five rats and are expressed as (treated/control) × 100 ± SEM. The enzyme activities in control rats were 49 ± 4 μmol *p*-hydroxyphenylpyruvate/g liver/hr, 2·6 ± 0·2 μmol kynurenine/g liver/hr, 251 ± 27 μmol PO_4/g liver/hr, and 152 ± 10 μmol PO_4/g liver/hr for TAT, TPO, glucose-6-phosphatase and fructose-1,6-diphosphatase respectively; the cytochrome *P*-450 level in the controls was 14·8 ± 2·6 nmol/g liver.

Table 1. *Protein synthesis and hepatic enzyme activity in rats fed on diets containing 1 or 5% caprolactam for 7 days and in pair-fed controls given an equal amount of rat chow*

Treatment group	Protein synthesis (dpm [^{3}H]leucine/mg protein)	Enzyme activity†			
		TAT	TPO	G6P	F1, 6DP
Caprolactam (1%)	799 ± 73	43·9 ± 4·2	1·8 ± 0·2*	264 ± 8	107 ± 4
Pair-fed controls	879 ± 73	53·9 ± 6·1	0·7 ± 0·1	220 ± 1	102 ± 6
Caprolactam (5%)	832 ± 165*	66·7 ± 6·8	2·2 ± 0·4	211 ± 13	99 ± 5*
Pair-fed controls	1441 ± 189	94·4 ± 19	3·8 ± 0·9	224 ± 10	120 ± 4

†TAT = Tyrosine aminotransferase (μmol *p*-hydroxyphenylpyruvic acid/g liver/hr). TPO = Tryptophan oxygenase (μmol kynurenine/g liver/hr). G6P = Glucose 6-phosphatase (μmol phosphate/g liver/hr). F1, 6DP = Fructose 1,6-diphosphatase (μmol phosphate/g liver/hr).

Rats were given the experimental diet for 7 days and killed on day 8. Enzyme activities and levels of protein synthesis were determined in liver homogenates. Values are means ± SEM for groups of five animals and those marked with an asterisk differ significantly (Student's *t* test) from the control values: $*P < 0{\cdot}05$.

Effects of repeated injections

The effects of daily ip injections of 500 mg caprolactam/kg on liver-enzyme activities are shown in Fig. 3. TAT activity increased sharply within 6 hr of the first injection to 320% of the control level. However, 6 hr after the second and third injections, TAT activities were only 200 and 225% of control levels, respectively, and after the eighth injection TAT activity was not significantly different from the control. Similarly, the increase in TPO activity diminished as the number of injections increased; after injections 1, 2 and 3, TPO activity was 415, 365 and 190% of control, respectively, and after the eighth injection, TPO activity did not differ from the control level. Neither glucose-6-phosphatase nor fructose-1,6-diphosphatase activity was markedly affected by repeated injections of caprolactam, but cytochrome *P*-450 levels were suppressed to 65, 65 and 50% of control levels after injections 1, 2 and 3 and had returned to control levels after the eighth injection.

Paired-feeding study

The results of studies on liver-enzyme activities after paired feeding of caprolactam to rats for 7 days are shown in Table 1. The food intake of caprolactam-exposed rats was determined and control rats were fed the same weight of diet as that consumed by caprolactam-exposed rats. A slight yet statistically significant induction of TPO was observed with 1% caprolactam, but not with 5%. Feeding 1% caprolactam did not alter [^{3}H]leucine incorporation into liver proteins. However, food restriction (comparing the pair-fed controls) caused a 63% increase in [^{3}H]leucine incorporation into protein. The inclusion of caprolactam in the diet blocked the stimulation of protein synthesis caused by food restriction. The body-weight gains and liver weights for these groups are presented in Table 2, which shows that feeding 5% caprolactam led to a marked reduction in food conversion efficiency, in comparison with the corresponding controls.

Effects of adrenalectomy and inhibitors on caprolactam induction

In order to determine whether endogenous steroids were required for caprolactam induction, the effects of caprolactam on enzyme induction in adrenalectomized rats were determined (Table 3). Administration of 1·0 or 1·5 g caprolactam/kg to adrenalectomized rats caused statistically significant increases in the activities of TAT in liver homogenates. Simultaneous administration of actinomycin D (1 mg/kg) and caprolactam (1·0 or 1·5 g/kg) increased the toxic effects of caprolactam but had no effect on TAT induction; TAT activity did not differ from that in rats treated only with caprolactam, but was significantly higher than that in those given only actinomycin D. This suggested that RNA synthesis was not necessary for caprolactam-mediated enzyme induction.

The results of studies on the effects of actinomycin D in intact rats are shown in Table 4. Actinomycin D suppressed the induction of TAT activity by hydrocortisone, but only partially suppressed induction of this enzyme by tryptophan or caprolactam. The

Table 2. *Effects of feeding caprolactam to rats for 7 days: comparison with pair-fed controls*

Treatment	Weight gain (g)	Liver weight (g)	Relative liver weight (liver weight/ 100 g body weight)	Food conversion efficiency (g weight gained/ g diet consumed)
Caprolactam (1%)	27 ± 4	5·2 ± 0·2	4·9 ± 0·2	35
Pair-fed controls	16 ± 1	5·1 ± 0·1	4·9 ± 0·2	30
Caprolactam (5%)	−23 ± 2	2·9 ± 0·3	4·5 ± 0·5	−146
Pair-fed controls	−8 ± 3	2·4 ± 0·1	3·3 ± 0·2	−33

Rats were given diets containing 1 or 5% caprolactam for 7 days. Their daily food intake was calculated and control rats were given an equal amount of untreated rat chow. The mean food intake of the 1 and 5% groups was 11·1 and 2·3 g/day, respectively, and the calculated dose of caprolactam was 1·1 and 1·8 g/kg/day, respectively. Values are means ± SEM for groups of five rats.

Table 3. *Effect of actinomycin D on the hepatic induction of tyrosine aminotransferase by caprolactam in adrenalectomized rats*

Treatment	Dose (mg/kg)	No. of animals	Tyrosine aminotransferase activity (μmol *p*-HPP/g liver/hr)
Control	0	9	30 ± 4
Caprolactam	1000	8	57 ± 5*
	1500	2	63 ± 0*
Actinomycin D	1	5	42 ± 4
Caprolactam + actinomycin D	1000 + 1	3	59 ± 2*†

p-HPP = *p*-Hydroxyphenylpyruvic acid

Rats were adrenalectomized at least 7 days before treatment. Caprolactam was given orally and actinomycin D was administered ip. The animals were killed 6 hr after treatment and enzyme activity was determined in liver homogenates. Values are means ± SEM for the numbers of animals shown, and those marked with a superscript differ significantly (Student's *t* test) from the control values (*$P < 0.01$) or from those for the group given actinomycin D alone (†$P < 0.05$).

enzyme activities in both the actinomycin D plus caprolactam-treated rats and the actinomycin D plus tryptophan-treated rats were greater than in controls or in rats treated with actinomycin D alone.

Effects of aflatoxin B_1.

In order to confirm the effects of actinomycin D, the effects of aflatoxin B_1 on caprolactam induction of TAT were determined (Table 5). As with actinomycin D, aflatoxin B_1 only partially blocked the induction of TAT activity by caprolactam. The induction by caprolactam in the presence of aflatoxin B_1 was significantly greater than in the controls.

DISCUSSION

The data recorded here show that caprolactam increased the activity of TAT and TPO. This response was rapid, occurring within 3 hr, and lasted for approximately 24 hr after administration of a single dose. Preliminary studies (A. J. Salerno, unpublished data 1979) have indicated that the effect of caprolactam is an induction rather than a stimulation of enzyme activity since continued enzyme-protein synthesis is required. Cycloheximide, an inhibitor of protein synthesis, blocked the caprolactam effects. In the present studies, the induction response was dose dependent, with no-effect levels of 300 mg caprolactam/kg for induction of TAT and of less than 300 mg caprolactam/kg for induction of TPO. These increases in enzyme activity were selective; the activities of enzymes involved in gluconeogenesis (i.e. glucose-6-phosphatase and fructose-1,6-diphosphatase) were not increased by caprolactam treatment. That some form of metabolic adaptation took place in caprolactam-treated rats was indicated by the reversal of the initial increase in the levels of enzyme activity when the animals were given repeated daily injections of caprolactam. After eight injections there was no detectable enzyme induction.

Table 4. *Effect of actinomycin D on the hepatic induction of tyrosine aminotransferase by caprolactam, hydrocortisone and tryptophan in rats*

Treatment	No. of animals	Tyrosine aminotransferase activity* (μmol *p*-HPP/mg protein/hr)
Control	5	$115 \pm 12^{a,b}$
Caprolactam	5	300 ± 23^{c}
Actinomycin D	5	78 ± 5^{e}
Actinomycin D + caprolactam	4	$156 \pm 9^{b,c,d}$
Hydrocortisone acetate	5	398 ± 22^{f}
Actinomycin D + hydrocortisone acetate	4	$132 \pm 6^{a,b,c}$
Tryptophan	4	478 ± 41^{g}
Actinomycin D + tryptophan	5	$212 \pm 11^{c,d}$

p-HPP = *p*-Hydroxyphenylpyruvic acid

*Means ± SEM for the numbers of animals shown. Means not carrying a common superscript differ significantly ($P < 0.05$) by the *Q* method with one-way classification analysis of variance.

Groups of rats were given ip injections (1 mg/kg) of actinomycin D or its solvent (5% aqueous ethanol) and 1 hr later were treated with caprolactam (1·5 g/kg, orally), hydrocortisone acetate (150 mg/kg, ip), tryptophan (600 mg/kg, ip) or distilled water. The rats were killed 7 hr after administration of actinomycin D and enzyme activity in liver homogenates was determined.

Table 5. *Effect of aflatoxin B_1 on induction of tyrosine aminotransferase by caprolactam*

Treatment	Tyrosine aminotransferase activity (μmol *p*-HPP/g liver/hr)
Control	22·6 ± 4·3
Aflatoxin B_1 (3 mg/kg)	29·3 ± 4·9
Caprolactam (1·5 g/kg)	92·7 ± 9·8*†
Caprolactam (1·5 g/kg) + aflatoxin B_1 (3 mg/kg)	46·3 ± 7·9*

p-HPP = *p*-Hydroxyphenylpyruvic acid

Rats were given oral doses of caprolactam in combination with ip injections of either aflatoxin B_1 or its solvent, DMSO. The rats were killed 6 hr later, and the livers were removed and assayed for enzyme activity. Values are means ± SEM for groups of five animals and those marked with superscripts differ significantly (Student's *t* test) from the control values (*$P < 0.05$) or from those for the group given aflatoxin B_1 alone (†$P < 0.05$).

When control animals were pair fed to the same dietary intake as caprolactam-fed rats, the activities of these enzymes were essentially the same in both groups. However, the absolute enzyme activity was higher in groups fed the higher dietary level of caprolactam. Several findings unrelated to liver enzymology, but germane to caprolactam toxicology, were observed in this experiment (Table 2). Both groups of caprolactam-exposed rats consumed similar amounts of caprolactam, approximately 1·5 g/kg/day. In addition, the inclusion of 1% caprolactam in the diet had no effect on food conversion efficiency, but 5% caprolactam considerably impaired food conversion. This effect on food conversion efficiency was not reflected in liver size as the 5% caprolactam-fed rats had livers more closely resembling the 1% rats than their controls. Furthermore, caprolactam inhibited the adaptive changes in liver-protein synthesis that were associated with decreased food intake. Therefore it seems probable that high dietary levels of caprolactam can interfere with normal metabolic signals that cause adaptive changes in rodent-liver biochemistry (Table 1). When these adaptive changes are blocked, normal levels of protein or amino acids might not be conserved and this is shown by the decreased rates of liver-protein synthesis. Because of this the animals showed accentuated weight loss and very reduced food conversion efficiency.

The mechanism of caprolactam interference with the regulation of metabolic pathways is not clear. Study of the structure of caprolactam suggests that hydrolysis *in vivo* would convert it to ε-aminocaproic acid. If conversion were complete, the experimental animals could be considered to be consuming diets containing 1 or 5% of this non-essential amino acid. This high dietary intake might then cause an amino acid imbalance or some kind of toxicity that could eventually account for the observed biochemical abnormalities. Studies with actinomycin D support this hypothesis. Actinomycin D blocks RNA polymerase, a necessary component in the induction of TAT and TPO (Lin & Knox, 1958). For example, Table 4 shows the effect of actinomycin D on the hydrocortisone induction of TAT. However, actinomycin D did not completely block induction by caprolactam. On the contrary, the magnitude of the effect of actinomycin D on caprolactam induction was similar to its effect on tryptophan induction. Schimke *et al.* (1965) showed that the induction of TPO by tryptophan does not require an RNA polymerase component. The partial inhibition of caprolactam induction by actinomycin D may be explained by the action of endogenous steroids if injections of 1·5 g caprolactam/kg caused a stress resulting in adrenocortical steroid release. Induction of TAT in adrenalectomized rats was unaffected by actinomycin D.

Data presented in Fig. 3 and Table 1 suggest that adaptive changes take place after exposure to caprolactam, but the nature of these changes is not obvious. Figure 3 shows a decrease in the response after multiple caprolactam treatments. In rats the blood half-life of caprolactam after oral administration is approximately 5 hr (A. J. Salerno, unpublished data 1979). With this rapid excretion it seems unlikely that the adaptation mechanism could increase the rate of caprolactam excretion. However, since the activities of two enzymes involved in amino acid metabolism (TAT and TPO) are increased, it is probable that the activities of other enzymes that might facilitate the catabolism of caprolactam are also increased. On the other hand, the feeding of caprolactam for 7 days (Table 1) also caused an increase in enzyme activity, although when the animals were pair-fed the induction was not obvious. Further studies are necessary to clarify these observations.

The practical relevance of these findings in assessing risks and hazards to Nylon 6 workers is not clear. The dose levels required to induce these effects on liver enzymes are far in excess of those encountered in normal plant operations. Moreover, the observations reported here indicate that caution is necessary when extrapolating the results of routine studies of toxicology, teratology, reproduction and carcinogenicity at high dietary levels of caprolactam in animals to the effects of low-level exposure in man. The long-term consequences of abnormalities in animal metabolism and nutrition caused by high doses of caprolactam may be expected to involve secondary responses that are totally uncharacteristic of the toxicology of the test substance at or near use levels and that are not predictable from biochemical information on caprolactam. Furthermore, the participation of caprolac-

tam, at high doses, in normal biochemical pathways suggests that at low doses it would be devoid of toxicity. Further research is needed to determine the nature and extent of caprolactam participation in normal metabolic processes.

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THE INDUCTION OF RAT HEPATIC MICROSOMAL XENOBIOTIC METABOLISM BY *n*-OCTADECYL β-(3′,5′-DI-*TERT*-BUTYL-4′-HYDROXYPHENYL)-PROPIONATE

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Abstract—The hepatic effects of oral administration of *n*-octadecyl β-(3′,5′-di-*tert*-butyl-4′-hydroxyphenyl)propionate (OBPP) has been studied in male and female rats. OBPP administration produced both marked liver enlargement and the induction of a number of parameters of hepatic microsomal xenobiotic metabolism, including cytochrome *P*-450, mixed-function oxidase enzymes and UDPglucuronosyltransferase. Histological examination of liver sections from OBPP-treated rats revealed hypertrophy of the centrilobular cells of the liver lobule, and ultrastructural studies indicated a marked proliferation of the smooth endoplasmic reticulum. Reversibility studies demonstrated that the increase in liver size and in the activities of xenobiotic-metabolizing enzymes had substantially reverted to control levels 14 days after the cessation of OBPP treatment. It is concluded that OBPP is a potent inducer of hepatic xenobiotic metabolism in the rat, with properties similar to those of sodium phenobarbitone.

INTRODUCTION

The compound *n*-octadecyl β-(3′,5′-di-*tert*-butyl-4′-hydroxyphenyl)propionate (OBPP) is incorporated into a number of plastics materials as a thermal stabilizer and antioxidant. This paper presents the results of studies on the effect of OBPP administration on liver enlargement and the induction of xenobiotic-metabolizing enzymes in the male and female rat.

EXPERIMENTAL

Materials. OBPP was obtained from Ciba-Geigy AG, Basel, Switzerland. A white to light-yellow crystalline powder, m.p. 49–54°C, it was used as supplied. $NADP^+$, UDP glucuronic acid, DL-isocitric acid, Tris (tris(hydroxymethyl)aminomethane), 4-methylumbelliferyl β-D-glucuronide, bovine serum albumin (Fraction V), isocitrate dehydrogenase (EC 1.1.1.42, from pig heart) and β-glucuronidase (EC 3.2.1.31, from bovine liver) were purchased from the Sigma Chemical Co. Ltd., Poole, Dorset. All other reagents were of the highest purity available.

Animals and treatment. Male and female Wistar albino rats (60–80 g) were purchased from Scientific Agribusiness Consultants (International) Ltd., Occold, Suffolk (Laboratory Animal Centre accredited). The animals were caged in groups of five or six in accommodation maintained at 20 ± 2°C with a relative humidity of 30–70% and were allowed free access to Spratts' (Barking, Essex) Laboratory Diet Number 1 and water. After the rats had been acclimatized to the experimental regime for 5 days, OBPP was administered by daily gastric intubation at dose levels of 30, 100, 300 and 1000 mg/kg/day for 14 days. Control animals received corresponding quantities of the corn oil vehicle (10 ml/kg body weight/day). Animals were always dosed between 09.00 and 10.00 hr. Animals were weighed and killed 24 hr after the last dose of OBPP, except in the reversibility studies, in which rats were killed 7 or 14 days after treatment with either corn oil or 1000 mg OBPP/kg/day for 14 days. In some additional experiments, male rats were treated with either sodium phenobarbitone (80 mg/kg/day for 3 days) or 20-methylcholanthrene (20 mg/kg/day for 2 days).

Biochemical investigations. Animals were killed by cervical dislocation and the livers were excised, weighed and placed immediately into ice-cold 0·154 M-KCl containing 50 mM-Tris-HCl buffer (pH 7·4). All subsequent operations were performed at 0–4°C. Whole liver homogenates (0·25 g fresh tissue/ml) were prepared with a Potter-type Teflon-glass, motor-driven homogenizer (A. H. Thomas Co., Philadelphia, PA, USA). Homogenates were centrifuged at 10,000 **g** av. for 20 min and the postmitochondrial supernatant fractions were used for determinations of ethylmorphine *N*-demethylase (Holtzman, Gram, Gigon & Gillette, 1968), biphenyl 4-hydroxylase (Creaven, Parke & Williams, 1965) and UDPglucuronosyltransferase (EC 2.4.1.17) using 4-methylumbelliferone as the aglycone (Neale & Parke, 1973). Portions of the 10,000 **g** av. supernatant fractions were further centrifuged at 105,000 **g** av. for 60 min to sediment the microsomal fractions, which were assayed for cytochrome *P*-450 content according to the

method of Omura & Sato (1964). Whole homogenate- and microsomal-protein contents were estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard.

In some additional experiments, microsomal fractions were used for studies of ethyl isocyanide ligand binding and for investigations into the inhibition of 7-ethoxycoumarin *O*-deethylase. The interaction of ethyl isocyanide with cytochrome *P*-450 in dithionite-reduced microsomes was studied according to the method of Imai & Siekevitz (1971), the ratio of the Soret peaks at 455 and 430 nm being estimated at pH 7·4. The activity of 7-ethoxycoumarin *O*-deethylase was determined as described by Ullrich & Weber (1972), using metyrapone and 7,8-benzoflavone as inhibitors (Ullrich, Frommer & Weber, 1973).

Morphological investigations. Liver slices were fixed in neutral buffered formalin, and paraffin sections approximately 5 μm thick were cut and subsequently stained with haematoxylin and eosin. Pieces of liver tissue were processed for electron microscopy by fixing for 2 hr in 1% phosphate-buffered osmium tetroxide (pH 7·4) at 4°C and subsequently embedding in Araldite. Ultrathin sections were cut and double-stained with uranyl acetate and lead citrate.

RESULTS

Effect of OBPP administration on body weight and relative liver weight

The administration of OBPP orally for 14 days at dose levels of 30, 100 and 300 mg/kg/day had no effect on the body-weight gain of either male or female rats. Whilst 1000 mg OBPP/kg/day had no effect on the body-weight gain of male rats (Table 1), a significant reduction was observed in female rats (Table 2). OBPP treatment progressively stimulated the relative liver weight of male rats, the increases being statistically significant at all dose levels (Table 1). However, with female rats, an increase in relative liver weight was observed only at OBPP dose levels of 300 and 1000 mg/kg/day (Table 2).

Hepatic biochemical studies

The administration of OBPP to male rats at dose levels of 30–1000 mg/kg/day for 14 days significantly stimulated the microsomal cytochrome *P*-450 content and the activities of both of the mixed-function oxidase (mfo) enzymes measured (Table 1). At dose levels of 100–1000 mg/kg/day the activity of UDPglucuronosyltransferase and the microsomal-protein content were also significantly enhanced. OBPP treatment did not alter the wavelength maximum of the dithionite-reduced carbon monoxide absorption complex with microsomal cytochrome *P*-450 (data not shown). The treatment of female rats with OBPP produced effects comparable to those observed in male animals (Table 2). OBPP administration at any of the dose levels used had no effect on the total liver-protein content in either male or female rats (Tables 1 and 2).

Hepatic morphological studies

Histological examination of livers from either male or female rats treated with 300 mg OBPP/kg/day for 14 days revealed a slight hypertrophy of the centrilobular cells of the liver lobule. This effect was potentiated in animals receiving 1000 mg OBPP/kg/day. Ultrastructural examination of liver sections from control animals receiving corn oil orally for 14 days revealed no abnormalities in any of the intracellular organelles (Fig. 1). However, treatment with 300 mg OBPP/kg/day for 14 days resulted in a marked proliferation of the smooth endoplasmic reticulum (Fig. 2), although the appearance of all other intracellular organelles was similar to that of the controls.

Reversibility studies

In rats killed 7 days after the cessation of OBPP treatment (1000 mg/kg/day for 14 days), the relative liver weight, the microsomal cytochrome *P*-450 content and the activities of ethylmorphine *N*-demethylase, biphenyl 4-hydroxylase and UDPglucuronosyltransferase were still significantly elevated in both sexes (Table 3). Two weeks after the last dose of the test compound the activity of biphenyl 4-hydroxylase was still significantly enhanced in both sexes and the relative liver weight was above control levels in the male rats only. All the other parameters investigated had reverted to control levels in both sexes. Histological examination of livers from both male and female rats revealed no abnormalities either at day 7 or 14 after cessation of OBPP treatment.

Additional studies into the mechanism of induction of rat hepatic xenobiotic metabolism by OBPP

OBPP treatment (300 mg/kg/day orally for 14 days) had no significant effect on the ratio of the two Soret peaks of the interaction of ethyl isocyanide with dithionite-reduced microsomal preparations from male rats. The 455/430 nm peak ratios at pH 7·4 for corn oil (control) and OBPP-treated rats were 0·58 $\pm$ 0·03 and 0·63 $\pm$ 0·06, respectively (mean $\pm$ SEM of ten animals in each case).

Microsomal preparations from control, sodium phenobarbitone-, 20-methylcholanthrene- and OBPP-treated male rats (the latter group being given 300 mg OBPP/kg/day orally for 14 days) were used for studies on the inhibition of 7-ethoxycoumarin *O*-deethylase activity by either metyrapone or 7,8-benzoflavone. Metyrapone, an inhibitor of cytochrome *P*-450-dependent mfo enzyme activities (Goujon, Nebert & Gielen, 1972; Ullrich *et al.* 1973) markedly inhibited 7-ethoxycoumarin metabolism when added at concentrations of either 10 or 100 μM to microsomal preparations from control, sodium phenobarbitone-treated or OBPP-treated animals (Fig. 3). In contrast, 10 μM-metyrapone had no effect on enzyme activity in microsomal preparations from 20-methylcholanthrene-treated animals. In concentrations of 1 or 10 μM, 7,8-benzoflavone, an inhibitor of cytochrome *P*-448-dependent mfo enzyme activities (Ullrich *et al.* 1973; Wiebel, Leutz, Diamond & Gelboin, 1971) markedly inhibited 7-ethoxycoumarin *O*-deethylase activity in microsomal preparations from 20-methylcholanthrene-treated rats, whilst stimulating enzyme activity in microsomal fractions from control, sodium phenobarbitone-treated and OBPP-treated animals (Fig. 3).

DISCUSSION

The results of this study demonstrate that OBPP treatment markedly stimulates both liver size and a number of parameters of hepatic xenobiotic metabo-

Table 1. *The effect of OBPP administration on the body weight, relative liver weight and some parameters of hepatic xenobiotic metabolism in male rats*

Parameter	Dose (mg/kg/day)...	Mean values				
		0	30	100	300	1000
Terminal body weight (g)		186 ± 6	184 ± 7 (100)	187 ± 6 (100)	175 ± 7 (95)	169 ± 8 (90)
Relative liver weight (g/100 g body weight)		5·0 ± 0·1	5·6 ± 0·1** (110)	6·0 ± 0·2*** (120)	6·9 ± 0·1*** (140)	7·9 ± 0·3*** (160)
Total liver protein (mg/g liver)		178 ± 7	183 ± 12 (105)	191 ± 5 (105)	178 ± 10 (100)	192 ± 7 (110)
Microsomal protein (mg/g liver)		27·4 ± 1·6	28·2 ± 1·0 (105)	37·7 ± 2·5*** (140)	33·5 ± 2·0* (120)	45·6 ± 1·7*** (165)
Cytochrome *P*-450 (ΔE 450–490 nm/mg microsomal protein)		0·072 ± 0·004	0·103 ± 0·004*** (145)	0·143 ± 0·006*** (200)	0·166 ± 0·004*** (230)	0·198 ± 0·006*** (275)
Ethylmorphine *N*-demethylase (μmol product/hr/g liver)		16·2 ± 1·0	24·1 ± 2·1** (150)	41·4 ± 4·1*** (255)	51·0 ± 2·5*** (315)	72·3 ± 3·4*** (445)
Biphenyl 4-hydroxylase (μmol product/hr/g liver)		3·9 ± 0·2	6·5 ± 0·3*** (165)	10·0 ± 0·4*** (255)	10·0 ± 0·4*** (255)	9·6 ± 0·5*** (245)
4-Methylumbelliferyl glucuronosyl-transferase (μmol product/hr/g liver)		82 ± 17	108 ± 3 (130)	170 ± 16** (205)	169 ± 15** (205)	229 ± 20*** (280)

Rats were treated by daily gastric intubation with either corn oil (control) or OBPP at the dose levels indicated for 14 days. All results are expressed as the mean ± SEM of either six control or five OBPP-treated animals, the percentage changes from control values being shown in brackets. Asterisks indicate results significantly different (Student's *t* test) from the control: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 2. *The effect of OBPP administration on the body weight, relative liver weight and some parameters of hepatic xenobiotic metabolism in female rats*

Parameter	Dose (mg/kg/day)...	Mean values				
		0	30	100	300	1000
Terminal body weight (g)		150 ± 5	148 ± 5 (100)	154 ± 3 (100)	141 ± 4 (95)	120 ± 6** (80)
Relative liver weight (g/100 g body weight)		4·8 ± 0·2	4·8 ± 0·2 (100)	5·1 ± 0·1 (105)	6·0 ± 0·1** (125)	7·0 ± 0·5** (145)
Total liver protein (mg/g liver)		189 ± 7	193 ± 6 (100)	193 ± 4 (100)	202 ± 7 (105)	203 ± 8 (105)
Microsomal protein (mg/g liver)		36·4 ± 0·9	37·5 ± 2·3 (105)	42·2 ± 1·6** (115)	43·1 ± 1·5** (120)	58·6 ± 2·6*** (160)
Cytochrome *P*-450 (ΔE 450–490 nm/mg microsomal protein)		0·058 ± 0·002	0·080 ± 0·006** (140)	0·089 ± 0·003*** (155)	0·141 ± 0·004*** (245)	0·168 ± 0·005*** (290)
Ethylmorphine *N*-demethylase (μmol product/hr/g liver)		8·2 ± 0·7	15·4 ± 3·6** (190)	20·9 ± 2·2*** (255)	39·2 ± 1·4*** (480)	35·0 ± 2·3*** (425)
Biphenyl 4-hydroxylase (μmol product/hr/g liver)		3·5 ± 0·3	5·9 ± 0·8*** (170)	9·9 ± 0·4*** (285)	11·2 ± 0·5*** (320)	10·3 ± 0·3*** (295)
4-Methylumbelliferyl glucuronosyl-transferase (μmol product/hr/g liver)		84 ± 10	114 ± 7* (135)	116 ± 11* (140)	116 ± 16* (140)	144 ± 16** (170)

Rats were treated by daily gastric intubation with either corn oil (control) or OBPP at the dose levels indicated for 14 days. All results are expressed as the mean ± SEM of either six control or five OBPP-treated animals, the percentage changes from control values being shown in brackets. Asterisks indicate results significantly different (Student's *t* test) from the control: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

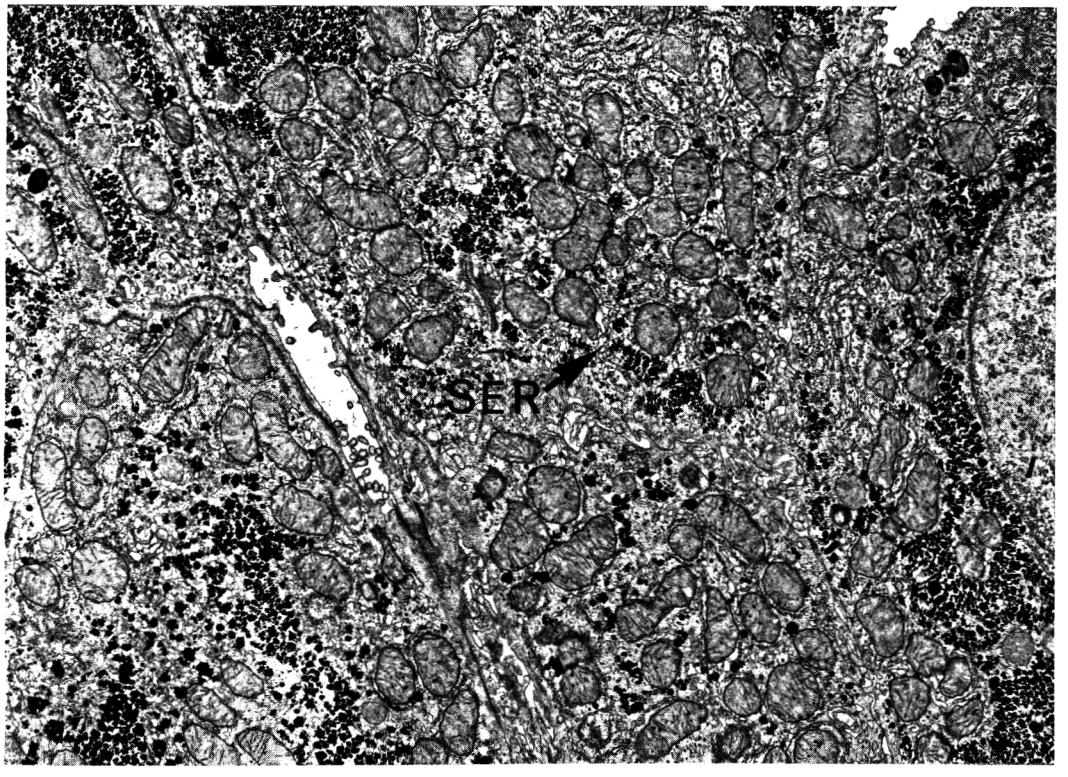

Fig. 1. Ultrastructure of the liver of a control rat treated orally with 10 ml corn oil/kg/day for 14 days. All the intracellular organelles appear normal. × 9000. SER = Smooth Endoplasmic Reticulum.

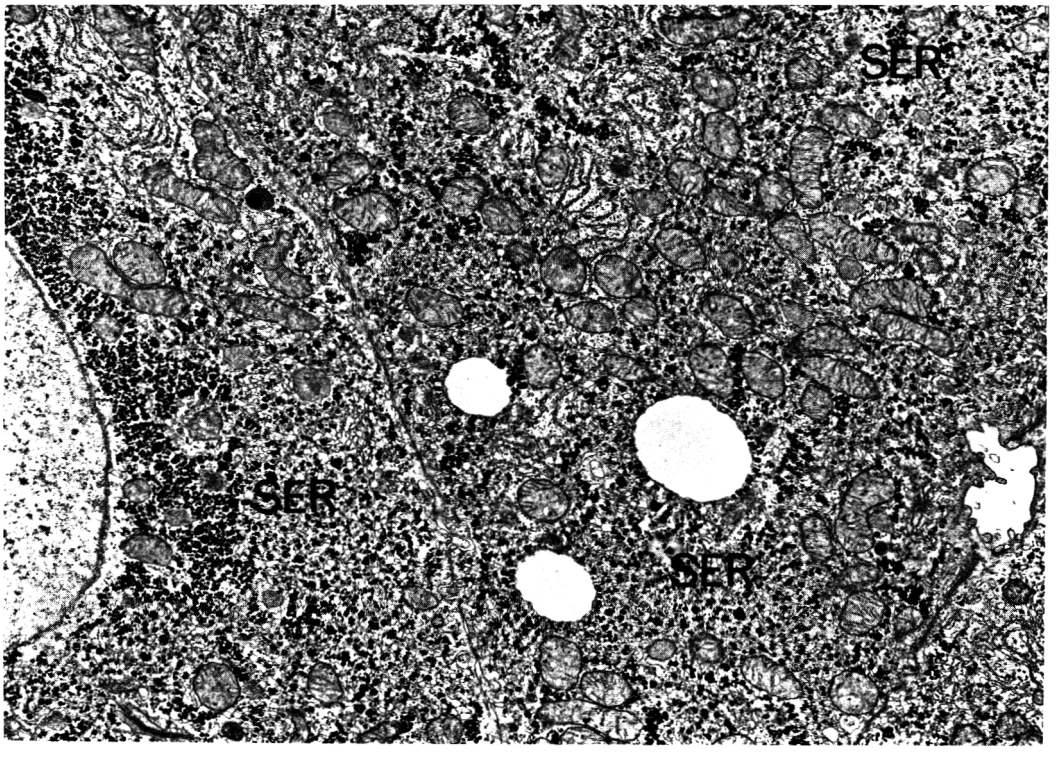

Fig. 2. Ultrastructure of the liver of a rat treated orally with 300 mg OBPP/kg/day for 14 days, showing a marked proliferation of the smooth endoplasmic reticulum (SER). All the other intracellular organelles are similar in appearance to those of control animals. × 9000.

Table 3. *The reversibility of the hepatic effects of OBPP administration in male and female rats*

Parameter	Values for OBPP-treated rats (% of control)			
	7†		14†	
	Males	Females	Males	Females
Relative liver weight	110*	110*	110*	100
Total liver protein	105	105	95	105
Microsomal protein	100	105	100	105
Cytochrome *P*-450	115*	115*	95	95
Ethylmorphine *N*-demethylase	150**	120*	90	90
Biphenyl 4-hydroxylase	135**	154**	115*	125*
4-Methylumbelliferyl glucuronosyltransferase	120*	130*	105	100

†No. of days after cessation of treatment.

Rats were treated with either corn oil (controls) or 1000 mg OBPP/kg/day by daily gastric intubation for 14 days. All results are for groups of six corn oil-treated and six OBPP-treated rats. Asterisks indicate results differing significantly (Student's *t* test) from the control: $*P < 0.05$; $**P < 0.01$.

lism in male and female rats. The induction of cytochrome *P*-450 and mfo enzyme activities was associated with the proliferation of the smooth endoplasmic reticulum of the hepatocytes, thus leading to increased microsomal-protein content. Indeed, the morphological changes observed as a consequence of OBPP treatment, namely the hypertrophy of the centrilobular cells of the liver lobule and the ultrastructural changes, were very similar to those observed after the administration of mfo-enzyme inducers such as sodium phenobarbitone (Crampton, Gray, Grasso & Parke, 1977; Fouts & Rogers, 1965; Stäubli, Hess & Weibel, 1969; Wright, Potter, Wooder, Donninger & Greenland, 1972). However, the findings of the reversibility study demonstrated that the cessation of OBPP treatment was followed by a substantial regression of the observed hepatic effects of the compound within 14 days, in both male and female rats.

Whilst many foreign compounds are known to stimulate hepatic xenobiotic-metabolizing enzyme activities, most have been classified into two main classes, namely those of the drug type, as exemplified by sodium phenobarbitone, and those of the polycyclic hydrocarbon type, such as 20-methylcholanthrene (Conney, 1967). Thus sodium phenobarbitone induces microsomal cytochrome *P*-450, whilst 20-methylcholanthrene treatment leads to the formation of cytochrome *P*-448 (Alvares, Schilling, Levin & Kuntzman, 1967). From the present studies the characteristics of the xenobiotic-metabolizing enzymes induced by OBPP treatment indicate that this compound should be assigned to the sodium phenobarbitone class of enzyme inducers. For example, the wavelength maximum of the dithionite-reduced carbon monoxide absorption complex with hepatic microsomes remained at 450 nm after OBPP treatment and there

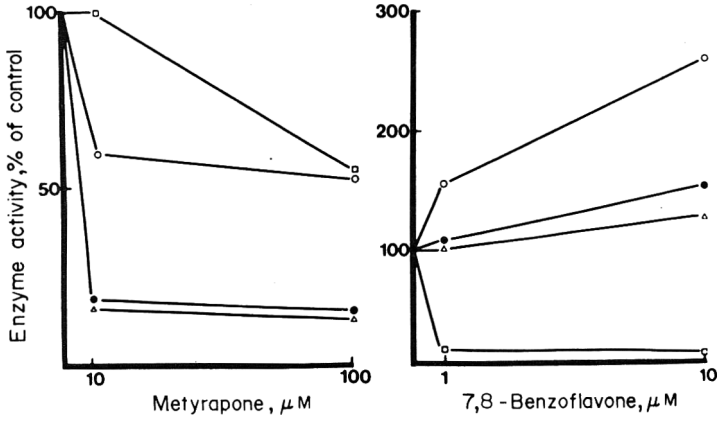

Fig. 3. Inhibition of microsomal 7-ethoxycoumarin *O*-deethylase by metyrapone and 7,8-benzoflavone. Pooled microsomal fractions (four rats/group) were prepared from control rats (○), or from rats treated with 300 mg OBPP/kg/day orally for 14 days (●), with 80 mg sodium phenobarbitone/kg/day ip for 3 days (△), or with 20 mg 20-methylcholanthrene/kg/day ip for 2 days (□). Enzyme determinations were performed at 30°C in incubation mixtures consisting of 0·1 mM 7-ethoxycoumarin, 0·15 mM NADPH and microsomes (approximately 0·1 mg protein/ml) in 0·1 M Tris–HCl buffer (pH 7·4), together with different concentrations of inhibitor. Specific enzyme activities (in the absence of inhibitor) for untreated and OBPP-, sodium phenobarbitone- and 20-methylcholanthrene-treated rats were 0·7, 4·6, 4·0 and 9·5 nmol product/min/mg microsomal protein, respectively.

was aiso no alteration in the ratio of the two Soret peaks when ethyl isocyanide was used as a ligand for microsomal haemoprotein. Previous workers have demonstrated that 20-methylcholanthrene treatment, but not sodium phenobarbitone administration, substantially alters the nature of the interaction of both ligands with rat hepatic microsomal haemoprotein (Alvares, Bickers & Kappas, 1973; Alvares *et al.* 1967; Sladek & Mannering, 1969a). Similarly OBPP, like sodium phenobarbitone. induces the activity of rat hepatic ethylmorphine *N*-demethylase, whereas this mfo enzyme is refractory to stimulation by 20-methylcholanthrene (Lake, Longland, Gangolli & Lloyd, 1976; Sladek & Mannering, 1969b). In the mfo enzyme inhibition studies, the activity of 7-ethoxycoumarin *O*-deethylase from OBPP-treated rats was inhibited by the cytochrome *P*-450 inhibitor metyrapone (Goujon *et al.* 1972; Ullrich *et al.* 1973) but not by the cytochrome *P*-448 inhibitor 7,8-benzoflavone (Ullrich *et al.* 1973; Wiebel *et al.* 1971). Thus the results of the enzyme inhibition studies also suggest that OBPP belongs to the sodium phenobarbitone class of mfo-enzyme inducers.

One possible metabolic pathway of OBPP in the rat would be the enzymic de-esterification of the compound, yielding octadecan-1-ol and 3,5-di-*tert*-butyl-4-hydroxyphenylpropionic acid. This latter compound closely resembles the structure of the antioxidant butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene). BHT and a number of its analogues have been shown to produce both liver enlargement and induction of mfo-enzyme activity in the rat (Gilbert & Golberg, 1965; Gilbert, Martin, Gangolli, Abraham & Golberg, 1969). Furthermore BHT, like OBPP, appears to be a member of the sodium phenobarbitone class of mfo enzyme inducers as it stimulates hepatic ethylmorphine *N*-demethylase activity in the rat (Crampton *et al.* 1977; Lake *et al.* 1976). However, as there is a paucity of information on the structure–activity relationships determining the ability of a particular compound to induce hepatic mfo enzymes, we cannot speculate from the present data whether the observed enzyme induction after OBPP treatment is a consequence of the administration of the parent compound or is due to one or more of its metabolites.

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A TERATOLOGY STUDY OF TOPICALLY APPLIED LINEAR ALKYLBENZENE SULPHONATE IN RATS

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Abstract—Linear alkylbenzene sulphonate (LAS) was applied daily, during the gestation period, to the clipped skin of pregnant rats. Concentrations of 0·05, 0·1 and 0·5% active ingredient were applied and allowed to remain on the skin; concentrations of 1, 5 and 20% active ingredient were applied and removed after a 30-min exposure period. Three control groups were included in the study: one received no treatment, the second was clipped only, and the third was clipped and vehicle was applied to the exposed dermis by the method used for the continuous exposure groups. All females were killed at day 21 of gestation, the maternal rats were examined grossly, and the foetuses were examined in detail. The only effects attributed to LAS in this study were reduced body weights in the dams given the highest level and skin changes in the dams that received the two highest concentrations (5 and 20% for 30 min). These effects included erythema followed by skin thickening and fissuring. These observations were marked at the 20% level and slight at the 5% level. There were no findings indicative of effects of LAS on the foetal parameters evaluated. Specifically, there was no indication of teratogenic or embryotoxic effect from LAS.

INTRODUCTION

Linear alkylbenzene sulphonate (LAS) is a straight-chain anionic surfactant which has largely replaced the branched-chain alkylbenzene sulphonates (ABS) in detergent formulations (Nolen, Klusman, Patrick & Geil, 1975). Studies have demonstrated the lack of a teratogenic or embryopathic effect of LAS in mice, rats and rabbits following oral administration (Palmer, Readshow & Neuff, 1975a) or dermal application (Palmer, Readshow & Neuff, 1975b).

This investigation was conducted at the request of the Japan Soap and Detergent Association to confirm these earlier findings and to provide data on the teratogenic potential of topically-applied LAS in rats at higher dose levels than have previously been reported.

EXPERIMENTAL

Sexually mature virgin female Wistar rats, 12–18 weeks of age, were obtained from Charles River Breeding Laboratories, Wilmington, MA. Male Wistar rats used for breeding purposes were obtained from an in-house colony. All animals were housed individually and provided with food and water *ad lib.* Female rats selected for mating were placed with male rats nightly in a 1:1 ratio. Vaginal smears were taken each morning and the presence of sperm was considered a positive indication of mating. The day on which evidence of mating was observed was considered day 0 of gestation. Females which mated were assigned to one of nine treatment groups so that the day 0 mean group body weights were nearly equal. A total of 181 females were mated for this study (20 in groups I–VIII and 21 in group IX).

The test material (20·5% LAS, 0·2% alkylbenzene, 0·6% ash, 78·7% water, average alkyl chain length 11·7, average mol wt 344·0) was provided by the Japan Soap and Detergent Association.

Five to six hours prior to treatment an exposure site (roughly 24 cm^2) in the dorsothoracic region of each animal from groups II through IX was clipped to a length of 1 mm. The animals were reclipped every 48 hr throughout the study. Group I animals were unclipped, group II animals were clipped but not treated and group III animals were clipped and treated with tap water. The mated female rats were treated daily from day 0 through day 20 of gestation. A 0·5-ml sample of the appropriate concentration of LAS and/or tap water was applied once daily to the clipped area and spread with a gloved finger over as much of the exposure site as possible. Each application was carried out slowly over a 3-min period. In the 1, 5 and 20% LAS groups (groups VII, VIII and IX, respectively corresponding to 20, 100 and 400 mg/kg/day) the test material was allowed to remain on the backs of the animals for 30 min, after which it was removed with warm tap water. The test material was not removed from the backs of the animals in the 0·05, 0·1 and 0·5% LAS groups (groups IV, V and VI corresponding to 1, 2 and 10 mg/kg/day).

Animal body weight and food consumption were determined daily during the treatment period. Daily observations were also made for toxicological effects.

Table 1. *Reproductive data*

Group no.	No. of *corpora lutea*†	No. of implantation sites†	No. of live foetuses†‡		No. of resorbed foetuses†		Foetal weight (g)		Foetal sex ratio (M/F)§
			Male	Female	Early	Late	Male	Female	
I	14·3	11·3	5·1	6·0	0·20	0·05	5·24	4·97	0·84
II	13·2	12·0	6·1	5·3	0·50	0·10	5·32	5·07	1·15
III	13·3	12·1	5·9	5·7	0·45	0·10	5·25	4·98	1·04
IV	13·7	12·4	6·7	5·5	0·21	0·05	5·24	4·95	1·22
V	13·3	12·1	5·8	5·8	0·32	0·05	5·37	5·08	1·00
VI	13·6	12·5	6·0	6·1	0·35	0·05	5·24	5·03	0·99
VII	14·1	12·7	5·7	6·2	0·75	0	5·29	5·02	0·92
VIII	14·0	12·8	5·8	6·4	0·55	0·05	5·31	5·10	0·90
IX	13·1	12·2	6·6	5·1	0·47	0	5·24	4·95	1·29

*Only pregnant females that survived to day 21 and did not deliver early are included. The no. of females included was 20 for all groups except group IV which comprised 19 females.
†These values are means/dam.
‡There were no dead foetuses.
§These values were calculated from group totals.
There were no statistically significant differences between the groups for the parameters shown.

All dams were killed on day 21 of gestation using ethyl ether. The uterine horns were evaluated for the presence of implantation sites. The latter were further distinguished as viable or nonviable foetuses or resorption sites (early or late). The number of *corpora lutea* on each ovary was also recorded.

External examinations and sex determination were made for all foetuses. Half of the foetuses were prepared for skeletal examination using a modified method of Crary (1962), in which eviscerated foetuses were placed into an aqueous potassium hydroxide solution, stained with Alizarin red and placed in Mall's solution to remove excess stain prior to clearing and storage in benzyl alcohol–glycerine solution. The number of thoracic and lumbar vertebrae and phalanges (fore- and hind-limb) was recorded.

The other half of the foetuses were preserved in Bouin's solution and examined for neural and visceral defects by the free-hand, razor-blade sectioning technique of Wilson & Warkany (1965).

Statistical comparisons were made between groups I and II and between groups II and III; all the LAS-treated groups were compared to group III. The data were evaluated for statistical significance using the F-test and Student's t-test.

When variances differed significantly, Student's t-test was appropriately modified using Cochran's approximation (t'). Comparisons among groups were made where applicable by the chi-square method. All statistical methods were used as described by Snedecor & Cochran (1967). Probability levels of $P < 0{\cdot}05$ and $P < 0{\cdot}01$ were considered significant.

RESULTS AND DISCUSSION

Pregnancy rates were 100% in all groups except groups IV (95%) and IX (95·2%). No mortality was observed during the study.

Mean body weights for animals in group IX (20% LAS, 30-min exposure) were significantly reduced from days 12–21 of the gestation period (308 ± 20 g at day 21, in comparison with 325 ± 30 for controls). Other scattered differences in weight gain were not considered to reflect an effect of LAS at any dose level. Food consumption was comparable in all groups throughout gestation.

Table 2. *Mean number of completely ossified thoracic and lumbar vertebrae and phalanges*

Group	Vertebrae		Phalanges (bilateral)			
			Fore		Hind	
	Thoracic	Lumbar	Proximal	Distal	Proximal	Distal
I	12·6	6·0	5·3	9·9	0·1	9·8
II	12·2	6·0	5·1	9·9	0·2	10·0
III	12·6	6·0	5·2	9·8	0·1	9·9
IV	12·3	6·0	5·2	9·9	0·0	10·0
V	12·3	6·0	5·3	10·0	0·0	10·0
VI	12·5	6·0	5·4	10·0	0·1	10·0
VII	12·5	6·0	6·0	10·0	0·2	10·0
VIII	12·5	6·0	5·2	9·9	0·3	10·0
IX	12·4	6·0	5·3	9·9	0·4	10·0

Values are means/foetus and those for the test groups did not differ significantly from those for control group III.

Table 3. *Summary of foetal abnormality data for rats treated with LAS during pregnancy*

Abnormality / Group no...	No. of foetuses affected								
	I	II	III	IV	V	VI	VII	VIII	IX
Gross external examination									
No mouth, jaw underdeveloped, ears mispositioned posteriorly	1	0	0	0	0	0	0	0	0
Elongated snout, mandible protruding	1	0	0	0	0	0	0	0	0
No mouth, mandible incompletely formed	0	0	1	0	0	0	0	0	0
No anus	1	0	0	0	0	0	0	0	0
Kidney (L) small	0	0	1	0	0	0	0	0	0
Hindpaw (L) flexure defect	0	0	0	0	0	0	0	1	0
No skin covering caudal vertebrae	0	0	0	0	0	0	0	1	0
Small foetus (runt)	0	0	0	1	0	0	0	0	0
Slightly distended ureter	1	0	0	0	0	0	0	0	0
Haematoma	9	8	2	2	1	6	3	2	3
No. of foetuses (litters) evaluated...	221 (19)	228 (20)	231 (20)	231 (19)	234 (20)	241 (20)	238 (20)	243 (20)	232 (20)
Soft-tissue examination									
Ectopic kidney (L)	1	0	0	0	0	0	0	0	0
Ectopic testis	0	1	1	0	1	0	1	0	1
Slight hydrocephalus	0	0	0	0	0	0	1	0	0
Distended renal pelvis	12	6	4	4	4	7	8	2	11
Distended ureter	5	4	6	4	11	11	15	4	12
Distended bladder	3	7	1	3	9	0	8	7	1
No. of foetuses (litters) evaluated...	107 (19)	109 (20)	111 (20)	109 (19)	111 (20)	116 (20)	116 (20)	117 (20)	109 (20)
Skeletal examination									
Wavy ribs, nasal and frontal bones malformed premaxillia, maxilla and mandible small, skull 'bill-shaped'	1	0	0	0	0	0	0	0	0
Premaxilla unossified (absent), mandible shortened and malformed, nasal and frontals malformed, skull pointed, clavicles abnormally curved	1	0	0	0	0	0	0	0	0
Clavicles mispositioned	1	0	0	0	0	0	0	0	0
Nasals elongated, hyoid located in cervical region, mandible small and malformed	0	0	1	0	0	0	0	0	0
Small foetus, micromelia (all limbs), humerus (bilateral) malformed	0	0	1	0	0	0	0	0	0
Nasals elongated	0	0	0	1	0	0	0	0	0
Tail short	0	0	0	0	0	0	0	1	0
No. of foetuses (litters) evaluated...	114 (19)	119 (20)	120 (20)	122 (19)	123 (20)	125 (20)	122 (20)	126 (20)	123 (20)

Animals in the control groups and those in the groups continuously exposed to 0·05, 0·1 or 0·5% LAS did not exhibit any unusual cutaneous manifestations.

Slight skin discoloration in the form of light brown areas was noted in three rats from day 3 to 6 of gestation in group VII (1·0% LAS). In group VIII (5·0% LAS), 14 of 20 animals had slight erythema and dry skin from days 3 to 6 of gestation. There were also occasional (7 of 20) observations of slight skin thickening during this period. After day 6 erythema and fissuring were no longer evident. Brown discoloration continued to be observed in one or two animals throughout the gestation period.

At the highest dose level (Group IX), slight erythema was noted in most animals on days 2, 3 and 4 of gestation. After day 6 this reaction was no longer evident. Slight skin-thickening was recorded at the point of application in two animals on day 2 and was present in all animals by day 5. Moderate skin-thickening was occasionally noted in six animals during the first half of gestation. Slight fissuring was noted in 18 animals from day 4 until the end of gestation. Clear exudate and brown skin discoloration were also occasionally noted during this period in the high-dose group.

Statistically significant effects of LAS on mean number of *corpora lutea*, implantations, viable foetuses or resorptions were not found in any of the treatment groups. These data are summarized in Table 1. At autopsy the dams did not reveal any changes that were considered to be related to LAS exposure.

Foetal viability and size were comparable in all groups. Sex ratios exhibited commonly-observed variations and no relationship to LAS administration was evident at any treatment level.

The number of thoracic and lumbar vertebrae and phalanges (fore- and hind-limbs) were counted on the Alizarin-stained foetal specimens. These data were summarized in Table 2. There were no statistically significant differences in the mean number of ossified thoracic and lumbar vertebrae and phalanges between the LAS-treated groups and control group III.

The external, soft-tissue and skeletal evaluations of the foetuses are summarized in Table 3. Most skeletal malformations were noted in the control groups. Two pups in the untreated control group (I) and one in the water-treated group (III) had malformed nasal and frontal bones as well as mandibles; in two pups of the untreated control group (I) the maxillae and/or premaxillae were also malformed. Malformed nasal bones were also noted in one pup in the group (IV) that received the lowest concentration of LAS (0·05 mg/kg/day continuous exposure).

In the soft-tissue examination, distended renal pelvis, distended ureters, ectopic testis, and distended bladder were noted in litters from all groups. These and other isolated observations appeared to occur at random and were not considered to be related to LAS application. The skeletal and visceral abnormalities did not occur in a pattern indicating an effect of LAS administration, and were considered to be spontaneous.

This study was demonstrated that LAS is free of teratogenic and embryopathic effects when applied to the dermis of pregnant Wistar rats at concentrations that elicit marked skin changes and reductions in maternal body weight.

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Fd Cosmet. Toxicol. Vol. 18, pp. 59 to 64

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THE RELATIONSHIP OF INSOLUBLE NITRILOTRIACETATE (NTA) IN THE URINE OF FEMALE RATS TO THE DIETARY LEVEL OF NTA

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Abstract—Groups of seven female Fischer 344 and Charles River CD rats were given, for 42 and 30 days respectively, nitrilotriacetate (NTA) as $Na_3NTA.H_2O$ at dietary levels of 0·05, 0·1, 0·3, 0·5, 0·75 or 2%. NTA did not accumulate in the bladder tissues to any greater extent than in the heart and liver, even when urinary NTA levels were up to two hundred times those in the plasma. A small amount of insoluble NTA (as CaNaNTA) was present in the urine of rats fed 0·05 or 0·1% NTA. The source of this insoluble NTA, which was present at NTA levels at which CaNaNTA was soluble *in vitro*, was not identified. At greater dietary NTA levels, when urinary NTA exceeded 3 μmol/ml, there was a linear increase in the proportion of NTA in the crystalline form. When values were corrected for the level of insoluble NTA present in the urines of animals fed 0·05% NTA the 'true' point of spontaneous crystalluria was 0·3% dietary NTA. When these results were compared with those of two studies on NTA induction of bladder neoplasia there were strong indications of a causal relationship between NTA crystalluria and the induction of tumours in the urinary bladder.

INTRODUCTION

Nitrilotriacetate (NTA) is a urinary-tract toxin when fed at high levels to rats (Nixon, 1971; Anderson & Kanerva, 1978a; National Cancer Institute, 1977). The chronic ingestion of high doses of NTA is associated with the development of bladder neoplasms in female rats (National Cancer Institute, 1977). It has recently been suggested that the development of bladder neoplasms correlates with the presence of crystalline CaNaNTA in the urine (Anderson & Kanerva, 1978b).

The present report describes an experiment designed to provide a clearer picture of the relationship between dietary NTA concentration and the presence of insoluble NTA in the urine of female Charles River and Fischer 344 rats. The NTA concentration in bladder tissue was compared with that in other organs. These results are compared with the dose-response of bladder neoplasms reported in two chronic feeding studies.

EXPERIMENTAL

The animals used were young, mature females of two strains, Charles River CD and Fischer 344, and were respectively 67 and 75 days old when dosing began. Both strains of animals were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. They were housed in stainless-steel cages and kept at controlled temperature (70 ± 5°F) and humidity (40–60%) with a 12-hr light/dark cycle.

All diets containing unlabelled $NaNTA.H_2O$ were w/w mixtures of ground Purina Laboratory Chow (Ralston Purina Co. Inc., St. Louis, MO) and commercial $Na_3NTA.H_2O$ (Monsanto, St. Louis, MO). The animals were allowed food and distilled water *ad lib.* throughout the experiment. The labelled feed was prepared by adding a solution of [*carboxyl*-^{14}C]NTA at pH 11. After preparation the samples were assayed for total ^{14}C to determine homogeneity and dose of [^{14}C]NTA.

The Fischer and Charles River rats were given unlabelled $NaNTA.H_2O$ in their feed for 32 and 20 days, respectively, and then both strains were given feed containing [^{14}C]NTA for 10 days. NTA was administered at dose levels of 0·05, 0·1, 0·3, 0·5, 0·75 or 2%. Food intake and growth records were maintained on all animals throughout the experiment.

On days 5 and 13, when the animals were consuming the unlabelled NTA, 20-hr urine collections were made, and the urines were examined visually for the presence of crystalline CaNaNTA. The urines from animals in each dose group were pooled and their content of total insolubles was estimated by centrifuging at 700 **g** for 5 min using a Dynac (Clay Adams Inc., Parsippany, NJ) desk-top centrifuge. The pooled insolubles were suspended in 3 ml ethanol using a Vortex mixer (Lab-Line Instruments, Inc., Melrose Park, IL), then sedimented by centrifugation, air-dried for 24 hr, and weighed.

During the [^{14}C]NTA feeding, urines excreted over a 24-hr period were collected from each Fischer rat on days 5, 8 and 10 and from each Charles River rat on days 5 and 10. Each urine sample (unlabelled and labelled) was assayed with a Multistix (Ames Company, Elkhart, IN) and visually evaluated for blood and crystals. Its volume and pH were determined. The urines containing [^{14}C]NTA were diluted with water (1:24, v/v) for ^{14}C determinations. A 2-ml sample of each undiluted urine was filtered through a 0·45 μm Millipore (Millipore, Bedford, MA) filter and the filtrates were assayed for ^{14}C.

After ten days of [^{14}C]NTA ingestion, the animals were anaesthetized with ether, and blood was collected in heparinized syringes. The whole blood and the plasma obtained by centrifugation were assayed for total [^{14}C]NTA content by combustion of

Table 1. *Effect on dietary $Na_3NTA.H_2O$ on weight gain and food conversion efficiency in Charles River CD and Fischer 344 female rats*

Dietary level of $Na_3NTA.H_2O$ (%)	NTA intake (μmol/100 g/day)*		Weight gain (g) in		Food conversion efficiency (g weight gained/100 g food consumed) in	
	Charles River CD	Fischer 344	Charles River CD	Fischer 344	Charles River CD	Fischer 344
0	0	0	62 ± 4	32 ± 3	11·4 ± 0·7	7·5 ± 0·6
0·05	14·2	10·5	67 ± 6	41 ± 5	13·5 ± 1·2	9·7 ± 0·9
0·1	28·3	20·4	67 ± 4	39 ± 3	13·3 ± 0·7	9·8 ± 0·5
0·3	85·6	62·9	66 ± 3	35 ± 2	13·2 ± 1·5	8·5 ± 0·4
0·5	142	104	66 ± 2	34 ± 3	13·2 ± 0·3	8·5 ± 0·7
0·75	213	159	63 ± 5	38 ± 2	12·6 ± 0·8	9·1 ± 0·4
2·00	508	360	54 ± 3	13 ± 7	12·4 ± 0·4	3·3 ± 1·5

*Average NTA intake values over the entire NTA-exposure period, calculated from total diet intake and average body weight during NTA ingestion.
Charles River CD and Fischer 344 rats ingested NTA for 30 and 42 days respectively, and their initial mean body weights were 180 ± 1 g and 151 ± 1 g, respectively. Values are means ± SEM for groups of seven rats.

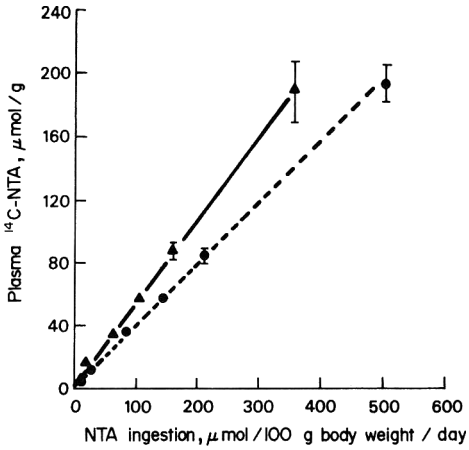

Fig. 1. The effect of ingestion of different levels of $Na_3[^{14}C]NTA.H_2O$ on the concentration of [^{14}C]NTA in the plasma of Fischer 344 (—▲—) and Charles River CD (--●--) rats. The values are means ± SEM for samples from groups of seven rats. The values were determined after 10 days ingestion of [^{14}C]NTA which was preceded by 42 or 30 days ingestion of unlabelled NTA by Fischer 344 or Charles River CD rats, respectively.

weighed samples to $^{14}CO_2$. The kidneys, heart, liver and urinary bladder were removed from three rats in each dose group for determination of their total ^{14}C content after combustion to $^{14}CO_2$. The bladders were opened, rinsed in tap water and blotted dry before weighing, and the other organs were rinsed in tap water and blotted dry.

The solubility of CaNa[^{14}C]NTA in control urines obtained from Sprague-Dawley and Charles River rats was determined. The urines were filtered through a 0·45 μm Millipore filter, then stirred with CaNa[^{14}C]NTA at room temperature for 21 hr. Samples of urine were taken at various times and total ^{14}C and soluble ^{14}C (0·45 μm Millipore filter) were determined.

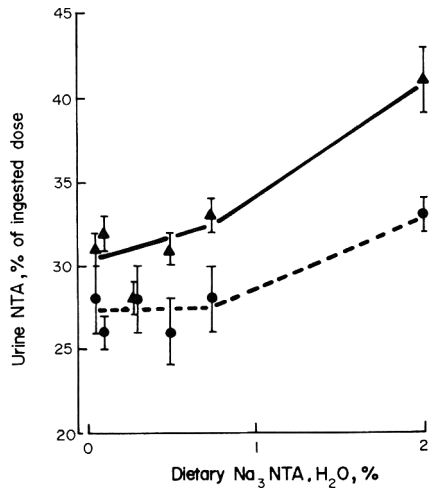

Fig. 2. The percentage of ingested [^{14}C]NTA recovered in the urine of female Fischer 344 (—▲—) and Charles River CD (--●--) rats given different dietary levels of $Na_3[^{14}C]NTA.H_2O$ for 10 days. Samples were taken from the Fischer 344 rats on days, 5, 8 and 10 and from the Charles River CD rats on days 5 and 10. Values are means ± SEM for all samples from groups of seven rats.

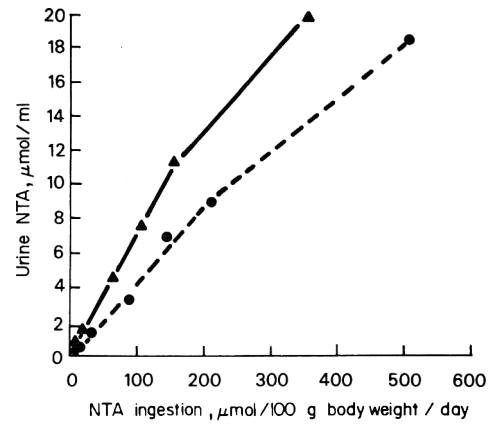

Fig. 3. The effect of ingestion of different levels of $Na_3[^{14}C]NTA.H_2O$ on the concentration of [^{14}C]NTA in the urine of Fischer 344 (—▲—) and Charles River CD (--●--) rats. Each value is the mean ± SEM for 21 or 14 determinations per NTA dose for Fischer 344 or Charles River CD rats, respectively.

RESULTS

The Charles River rats gained more weight and had greater food conversion efficiency than the Fischer animals, whether the feed contained NTA or not (Table 1). In both strains of animals, doses of up to 0·75% $Na_3NTA.H_2O$ in the diet resulted in small but consistent increases in weight gain and food conversion efficiency. $Na_3NTA.H_2O$ at 2% in the diet severely reduced weight gain and feed utilization in the Fischer rats. However, in Charles River rats given 2% NTA there was only a slight decrease in weight gain and the food conversion efficiency did not fall below the control value. The Charles River rats consumed on average 37% more NTA per 100 g body weight per day than the Fischer 344 rats. Plasma NTA concentrations were a linear function of NTA ingestion (μmol/100 g body weight/day) in both strains but the gradient of the dose-response line was greater for Fischer 344 rats than for Charles River rats (Fig. 1).

At all doses, Fischer 344 rats excreted a greater percentage of the ingested NTA in their urine than did the Charles River rats (Fig. 2). In both strains the amounts of ingested NTA that were excreted in the urine remained fairly constant at doses of up to 0·75% $Na_3NTA.H_2O$, but increased in both strains at the 2% dose level. At all doses the Fischer 344 rats had a higher urinary concentration of NTA than the Charles River rats (Fig. 3). This strain difference in urinary NTA concentration was more a consequence of a greater urinary output by the Charles River rats in all groups (Fig. 4) than due to a difference in disposition of dietary NTA. $Na_3NTA.H_2O$ consumption resulted in an increase in urine pH that was proportional to the $\log_{10}$ of excess Na ingestion (μmol/100 g body weight/day; Fig. 5).

The amount of insoluble NTA (that retained by a 0·45 μm filter) at the two lowest doses (0·05 and 0·1%) in both strains of rats was a small and roughly constant percentage (approx. 7%) of the total urinary NTA. When urinary NTA exceeded 3 μmol/ml there was a linear increase in the amount of insoluble NTA

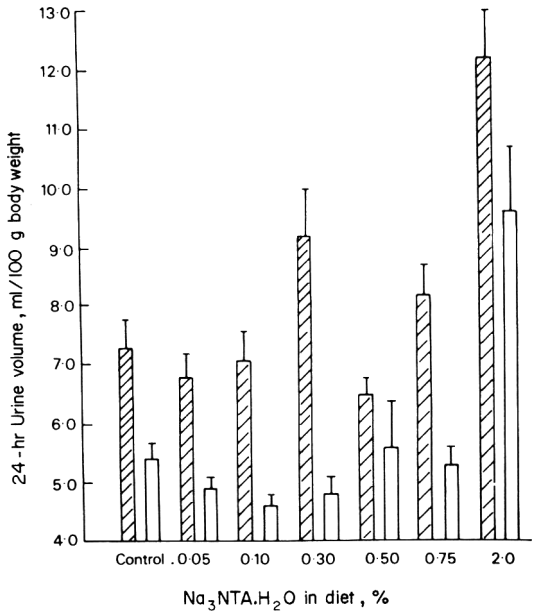

Fig. 4. The volume of urine excreted by Fischer 344 (□) and Charles River CD (▨) rats given different dietary levels of $Na_3NTA.H_2O$. Each value is the mean ± SEM for 21 or 14 determinations per NTA dose for Fischer 344 or Charles River CD rats, respectively.

with increasing total urinary NTA (Fig. 6). For reference the solubility of CaNa[^{14}C]NTA in rat urine *in vitro*, is indicated in Fig. 6. The extrapolated regression line for urinary insoluble [^{14}C]NTA (2·3 μmol/ml) is close to the upper limit of solubility determined *in vitro*.

Figure 7 depicts the ratios of the specific activities of the urine and of organs to the specific activity of the plasma at each [^{14}C]NTA dose. In both strains of animals the bladders had relative specific activities that were more reflective of plasma-NTA concentrations than of urinary-NTA concentrations, which were up to 200 times greater. In fact in both strains of rats, the liver and the kidneys showed greater tissue:plasma specific activity ratios than the bladder. These results show that even at very high doses of NTA, the bladder tissue does not accumulate NTA in proportion to the level in the urine. The higher relative NTA concentration in the urine of the Fischer 344 rats (about 200) compared with the Charles River rats (about 100) is a consequence of greater NTA absorption (Fig. 2), and smaller urine volume (Fig. 4).

DISCUSSION

The primary purpose of this work was to define the level of dietary NTA at which insoluble CaNaNTA begins to accumulate in the urine of female rats. Even the lowest dose of NTA that was fed (0·05%) resulted in detectable levels of NTA that were retained by a 0·45 μm filter. There are at least two possible sources of this insoluble NTA in the urine at levels at which CaNaNTA was shown to be soluble in experiments *in vitro*. It is possible that [^{14}C]NTA is adsorbed onto the non-specific insolubles detectable in rat urine. This material was shown to be independent of NTA dose in the diet, and to be present at a mean concentration of 1·47 ± 0·21 mg/ml of urine in 21 samples of urine from animals that were ingesting unlabelled NTA. Alternatively, the low background of insoluble NTA at low doses could also result from contamination of the urine samples with feed or faeces.

The contributions of these two possible sources were assessed experimentally. Non-specific adsorption was measured by adding [^{14}C]NTA (pH 7·4) to control urine, incubating it for 3 hr, and then filtering. Contamination by feed was measured by adding sufficient feed (23 mg feed/ml urine) to provide an amount of [^{14}C]NTA equivalent to the low background level of insoluble NTA. The urine was filtered after 3-hr incubation. In both of these experiments the amount of [^{14}C]NTA recovered in the filtrates was 100 ± 2% of the amount added. Thus neither of these possible sources of insoluble NTA appears to be significant.

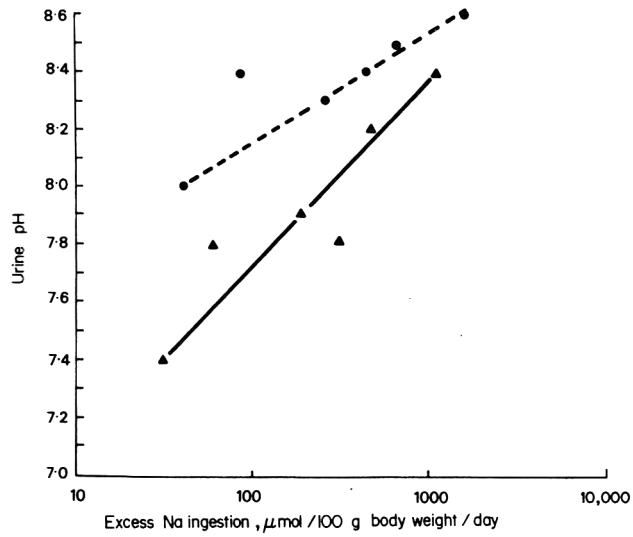

Fig. 5. Urinary pH in female Fischer 344 (—▲—) and Charles River CD (—●—) rats as a function of the log of dietary Na intake as $Na_3NTA.H_2O$.

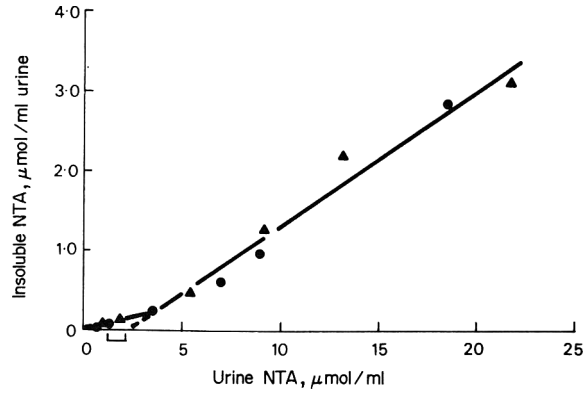

Fig. 6. Concentration of insoluble [^{14}C]NTA as a function of the concentration of total [^{14}C]NTA in the urine of female Fischer 344 (▲) and Charles River CD (●) rats. The regression line [$y = -0{\cdot}39 + 0{\cdot}17x$ ($r = 0{\cdot}99$)] has been extrapolated, and the solubility of CaNa[^{14}C]NTA in control rat urine is marked (⌴).

Even though the source of this small pool of insoluble NTA has not been identified we have corrected all values for the level observed in the urines from animals fed 0·05% $Na_3NTA.H_2O$ and this assessment of the 'true' point of spontaneous formation of insoluble NTA in female rat urine is presented in Fig. 8. The figure indicates that crystalluria begins when urinary NTA exceeds the load produced by a dietary level of 0·3% $Na_3NTA.H_2O$ (10·9 μmol/g feed; mol wt $Na_3NTA.H_2O$, 275). In Fig. 8 are also plotted the incidences of bladder tumours reported in an NCI bioassay (National Cancer Institute, 1977) and in a 2-yr feeding study in which female rats were fed NTA for 18 months or longer (Nixon, Buehler & Niewenhuis, 1972). The combined results of these studies show that among 140 female rats that consumed NTA at doses of up to 18 μmol NTA/g feed for 18 months or longer there was one bladder papilloma (in an animal fed 7·3 μmol $Na_3NTA.H_2O$/g feed). Above 18 μmol NTA/g feed there was a very rapid increase in urinary-bladder tumours.

The relationship between the two dose-response curves (Fig. 8) strongly suggests, but does not prove, that a causal relationship exists between crystalluria and the incidence of bladder neoplasia. If a causal relationship does exist it would make unreasonable any extrapolation of bladder tumorigenesis to NTA levels below those at which crystalluria exists.

The data in Fig. 7 show an additional significant factor concerning the relationship between NTA and bladder epithelial neoplasms. The bladder tissues are bathed in urine containing NTA at levels 100 to 200 times those of the plasma and yet they do not accumulate NTA to any greater extent than do the systemic organs (heart and liver) which are only in contact with plasma NTA. In fact, in all cases the bladders had lower NTA concentrations than the livers. The low NTA concentration in the bladder tissue relative to the urines from the same animals strongly suggests that the effects of NTA on bladder tissue are a consequence of the high NTA concentration in urine and are not dependent upon the tissue attaining high concentrations of the non-mutagenic NTA (Thayer & Kensler, 1973). In the light of this argument an indirect mechanism for tumorigenesis having no-effect levels seems likely.

It is important to relate the exposure levels of NTA associated with bladder damage to possible human exposure to NTA from drinking water. A recent publication of NTA analyses in ground waters reported by the Canadian government (Malaiyandi, Williams & O'Grady, 1979), reports the average NTA concentration in drinking water as 2·8 μg/litre. If a 50-kg person drank 2 litres of water containing this level of NTA each day, he would be exposed to 4×10^{-4} μmol NTA/kg/day. This value is only

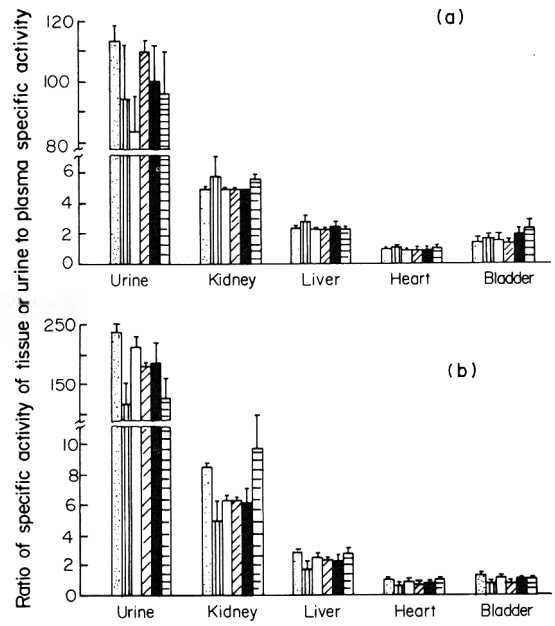

Fig. 7. Ratio of specific activity of tissue or urine to plasma specific activity in (a) Charles River CD and (b) Fischer 344 rats given 0·05 (▨), 0·1 (▥), 0·3 (□), 0·5 (▨), 0·75 (■) and 2% (▤) $Na_3[^{14}C]NTA.H_2O$ in the diet. Values are means ± SEM for groups of three animals.

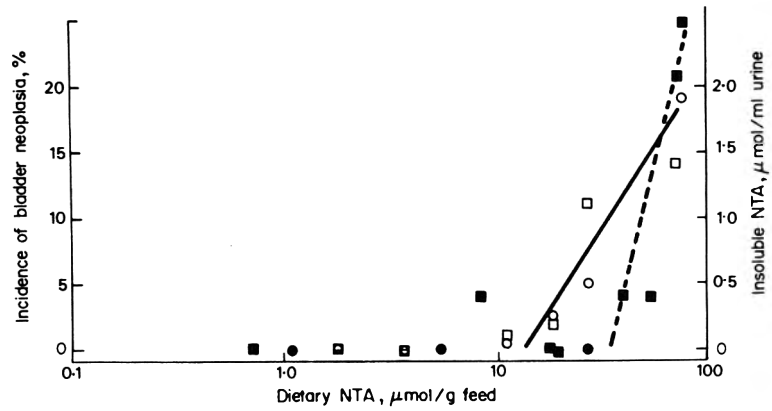

Fig. 8. The dose-response for bladder neoplasms in female rats fed NTA for 18 months or longer (---; Fischer 344, ■; Charles River CD, ●) compared with the dose-response for the concentration of insoluble NTA in the urine in the present short-term feeding study (——; Fischer 344, □; Charles River CD, ○).

1/262,500 or 1/355,000, respectively, of the levels to which the Fischer 344 or Charles River rats were exposed at the lowest (0·05%) NTA dose in this study. This very large difference in exposure levels makes it evident that it is virtually impossible for humans to be exposed to sufficient NTA to induce NTA crystalluria.

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EFFECT OF QUALITY AND QUANTITY OF DIET ON SURVIVAL AND TUMOUR INCIDENCE IN OUTBRED SWISS MICE

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Abstract—Groups of 80 mice of each sex of an outbred Swiss strain were fed either PRD or 41B (modified) diet either *ad lib.* or at 75% of the level consumed *ad lib.* The animals were fed these diets from an age of 4 wk and were observed daily, weighed regularly and given a full autopsy when they died. The experiment was ended after 18 months. In both sexes and on both types of diet, dietary restriction was associated with slightly better survival up to 18 months and with a highly significant decrease in the incidence of neoplasms of all types. At the same dietary level, tumour incidence was slightly but not significantly higher in mice fed the 41B diet than in those fed the PRD diet. These findings are discussed in relation to the interpretation of laboratory tests for carcinogenicity.

INTRODUCTION

The main purpose of the study was to define certain suitable conditions for the conduct of future carcinogenicity tests. We particularly wanted to know:

(i) whether one particular strain of outbred Swiss mice would constitute a suitable model in terms of longevity, freedom from disease, and 'spontaneous' incidence of tumours;

(ii) which of two commercially available laboratory animal diets, if either, would be more suitable and

(iii) whether there would be advantages in conducting future carcinogenicity studies under conditions of dietary restriction.

The third of these problems is regarded as important in view of recently reported findings (Nolen, 1972; Roe & Tucker, 1973; Tucker, 1979) and the earlier classical work (McCay, Crowell & Maynard, 1935; Tannenbaum, 1940, 1942 & 1945; Tannenbaum & Silverstone, 1949 & 1950). In all these reports, dietary restriction has been shown both to improve survival and to reduce the 'spontaneous' development of neoplasms in otherwise untreated rats or mice.

EXPERIMENTAL

Animals. Caesarian-delivered, barrier-maintained outbred Swiss mice were obtained from our own breeding unit at Kimpton, Herts. The colony was derived in 1963 from a breeding nucleus of Charles River CD mice but was rederived by Caesarian section in 1967. In our breeding unit mice are bred in pairs with the male not separated from the female at any time, with the result that *post-partum* mating occurs. The breeding mice are fed on PRD diet (see below) and provided with soft wood shavings as bedding.

Animal transport, caging, bedding and husbandry. The mice were weaned at 3 wk and transported in filtered transportation cages from the breeding unit to the animal house at our Research Laboratories at Welwyn Garden City. After arrival they were maintained under conventional conditions, four per cage initially, in polypropylene cages and provided with autoclaved soft wood shavings (obtained from W. P. Usher, Whetstone, London) for bedding. During the last 6 months of the 18-month experiment similar bedding was obtained from S.M.C. Ltd., Standon, Herts. Bedding was changed twice-weekly and a clean cage provided at 3-month intervals. Water was provided *ad lib.* to all animals throughout the study. The ambient temperature was kept at 20 ± 2°C and the relative humidity at 50 ± 5% with a 12-hr light–dark cycle.

Diets. The two diets compared were Porton Rat Diet (PRD) and 41B (modified). Both were obtained from Labsure Diets, Poole, Dorset, after irradiation by Irradiation Products Ltd., Wantage. The PRD diet was received in 15 batches at roughly monthly intervals and the 41B diet was received in ten batches at roughly two-month intervals. Specification and analytical details (at wk 1, 52 and 80) for the two diets are summarized in Table 1.

Pre-test observations and experimental design. On arrival 320 males and 320 females were allocated at random (using Table XXXIII Random Numbers, Fisher & Yates, 1963) to four groups each consisting of 80 males and 80 females and then weighed individually. During the next 7 days, mice were fed *ad lib.* on the diets they were scheduled to be given during the main study, their food consumption was measured and mice were individually ear-punched with a coded experimental number. On the seventh day (i.e. day 1 of the main study) all mice were weighed individually. Groups 1 and 2 were given the PRD diet and groups 3 and 4 received the 41B diet. The diets were fed *ad lib.* to groups 1 and 3 and restricted to 75% of the *ad lib.* level for groups 2 and 4. One female in group 2 became pregnant after escaping from its cage and was left out of the study.

Observations. Individual body weights and food consumption were measured weekly for 2 months,

Table 1. *Composition of test diets*

Constituent	Amount in PRD diet				Amount in modified 41B diet			
	Manufacturer's specifications	By analysis at...			Manufacturer's specifications	By analysis at...		
		Week 1	Week 52	End of test		Week 1	Week 52	End of test
Crude oil (%)	2·78	2·95	3·65	3·25	2·87	2·55	2·60	3·05
Crude protein (%)	19·79	20·70	21·50	20·50	16·61	19·25	18·40	18·25
Crude fibre (%)	5·37	4·75	5·05	4·15	5·60	5·90	5·75	4·70
Calcium (% as Ca)	0·72			0·60	1·19			0·90
Phosphorus (% as P)	0·71			0·77	0·79			0·85
Salt (% as NaCl)	1·03			1·11	0·52			0·70
Available energy (kcal/kg)	2570				2630			
Ash (%)		5·05	4·80	4·60		6·55	4·50	5·10
Moisture (%)		9·05	9·10	12·60		9·00	9·10	10·55
Trace Elements added (ppm)								
Cobalt	0·4				0·4			
Copper	7			12	7			14
Iodine	1·3				1·3			
Iron	30			232	30			284
Magnesium	102			1700	102			1600
Manganese	25			54	25			62
Vitamins added /kg of diet								
A (IU)	8000			6200	8000			4300
B_1 (mg)	2			10	2			10
B_2 (mg)	8			13	8			13
B_{12} (μg)	12			18000	12			19000
D_3 (IU)	1000				1000			
E (IU)	25			48	25			39
K (mg)	10				10			
Choline chloride (mg)	200			1850	200			2000
Folic acid (mg)	6			6·2	6			6·2
Nicotinic acid (mg)	20			69	20			83
Pantothenic acid (mg)	4			20	4			20
Amino acids added (%)								
Arginine	1·25			1·16	1·00			1·05
Cystine	0·27			0·25	0·22			0·22
Glycine	0·97				0·93			
Histidine	0·51			0·66	0·38			0·56
Isoleucine	0·88				0·68			
Leucine	1·58				1·16			
Lysine	1·07				0·80			
Methionine	0·36				0·31			
Phenylalanine	0·94				0·72			
Threonine	0·77			0·86	0·60			0·79
Tryptophan	0·25				0·18			
Tyrosine	0·76				0·56			
Valine	1·04				0·83			
Aflatoxin				ND				ND

ND = Not detected (<0·02 ppm)

fortnightly for 2 months and monthly thereafter. Food consumed by each cage of mice fed *ad lib.* (groups 1 and 3) was measured. These groups were given 50g quantities of pelleted food as necessary and the residues were weighed at intervals (detailed above) allowing the mean daily food consumption for each cage of mice to be calculated. The means for cages of mice of the same group were averaged to give the daily consumption by mice of each sex in groups 1 and 3. Mice in groups 2 and 4 were provided, between 9.30am and 10am each day, with 75% of the food consumed (calculated to the nearest whole gram) when given *ad lib.* to mice of the same sex on the same diet, based on the most recent estimate. Very little of either diet was wasted as a result of powdering and such powdering as occurred was ignored.

Ophthalmoscopy was carried out on 20 mice of each sex from each group after 4, 13 and 18 months by K. C. Barnett, M.A., PhD., F.R.C.V.S. Mice were inspected twice on each weekday, once on Saturdays, and once on Sundays, for general health. Sick mice were isolated, observed closely and weighed frequently. If they recovered they were returned to their original cages. If their condition deteriorated they were killed, one person (G.C.) having sole responsibility for deciding which and when animals in all groups should be killed. Mice were inspected and palpated for localized swellings on the same days as they were weighed.

Each mouse was given a full routine autopsy, except where this was limited by cannibalism or autolysis. The final body weight and liver weight were recorded. Organs showing pathological changes were fixed along with the following organs which were sampled routinely: liver, gall bladder, both kidneys, both adrenals, both seminal vesicles, both gonads, prostate or uterus, and paraffin wax sections stained with haematoxylin and eosin were prepared. One lung, one salivary gland, one cervical lymph node and one mesenteric lymph node were also sampled routinely and the brain and pituitary gland were taken from animals surviving until the end of the experiment. Sections were prepared from the brain, pituitary, cervical lymph node, mesenteric lymph node and salivary gland of a proportion of the animals. Sections were evaluated using standard parameters and systems for grading the severity of lesions where appropriate.

Termination of the study. A majority of mice in all groups survived for 80 wk. The survivors were killed between 80 and 83 wk, some mice of each group being killed on each day. Nineteen mice died spontaneously during this 3-wk period.

Statistical period. To compare the proportions of mice with particular lesions under two contrasting experimental conditions, the chi-square test for heterogeneity of proportions, after adjustment for stratification (Armitage, 1966) was used. The number of strata used in the analysis depended on the particular comparison being made. For example, comparing the incidence of tumours in mice killed at the end of the test, in the comparison of all *ad lib.* groups with all restricted groups, the analysis combined information from four strata (PRD Male; 41B Male; PRD Female; 41B Female). However, in comparing the total incidences of lung and liver tumours, time of death was used as an additional stratifying variable. The test carried out is thus similar to that described by Peto (1974), for incidental tumours, that is, tumours assumed not to have caused the death of the animal. (This was obviously true of animals terminally killed).

RESULTS

Food consumption

Food consumed by the groups fed *ad lib.* increased steeply to over 8g/day as mice grew to maturity during the first 10wk of the study (See Figs 1a and 1b). Between 12 and about 32 weeks food consumption fell again to plateau at between 6 and 7g/day for males and between 5 and 6g/day for females. Mice of both sexes tended to eat slightly more of the 41B diet than the PRD diet. Thus the amount of 41B diet consumed (group 3) exceeded the amount of PRD diet consumed (group 1) on 18/27 occasions in males and on 17/27 occasions in females, and the opposite was true on 8/27 occasions in males and 8/27 occasions in females. These differences were significant at the 99% confidence level.

Animals on restricted diets (groups 2 and 4) almost always consumed all the diet offered to them, and appeared hungry at feeding time each day. However, on some occasions when the ration was increased because the animals fed *ad lib.* had eaten more during the previous food consumption period, part of the ration remained uneaten 24-hr later. This occurred more often in the case of females than males and more often during the last 6 months of the study than earlier. In general the method for restricting mice of groups 2 and 4 to 75% of the food consumed by comparable mice fed *ad lib.* worked very well (see Figs 1a and b).

Survival

Mice of both sexes given restricted diets survived better than those fed *ad lib.* (Figs 2a and b). This difference was statistically significantly for females, ($P < 0{\cdot}05$), but was small and not significant for males. Mice on the restricted regime were also sleeker and more active than those fed *ad lib.* There was no obvious effect of type of diet (PRD or 41B) on survival.

Body weight and liver weight

Throughout the experiment mice fed *ad lib.* weighed more than those fed a restricted diet (see Figs 3a and b), the difference being highly significant ($P < 0{\cdot}01$) in both sexes from week 2 onwards. Mice on the PRD diet tended to weigh more than comparably fed mice on 41B diet. For mice fed *ab lib.* this difference was small and fairly consistent, though not significant for either sex. In mice fed restricted diets those on the PRD diets were significantly heavier ($P < 0{\cdot}001$) than those on the 41B diets at some times (e.g. wk 14 to 32). At other times the difference was not significant or even reversed. Table 2 shows the mean body, liver and relative liver weights for animals killed terminally in four groups after excluding animals with liver tumours or malignant lymphoma (i.e. conditions that affect the liver). Females fed *ad lib.* with both diets had significantly smaller relative liver weights than comparable females on restricted diets

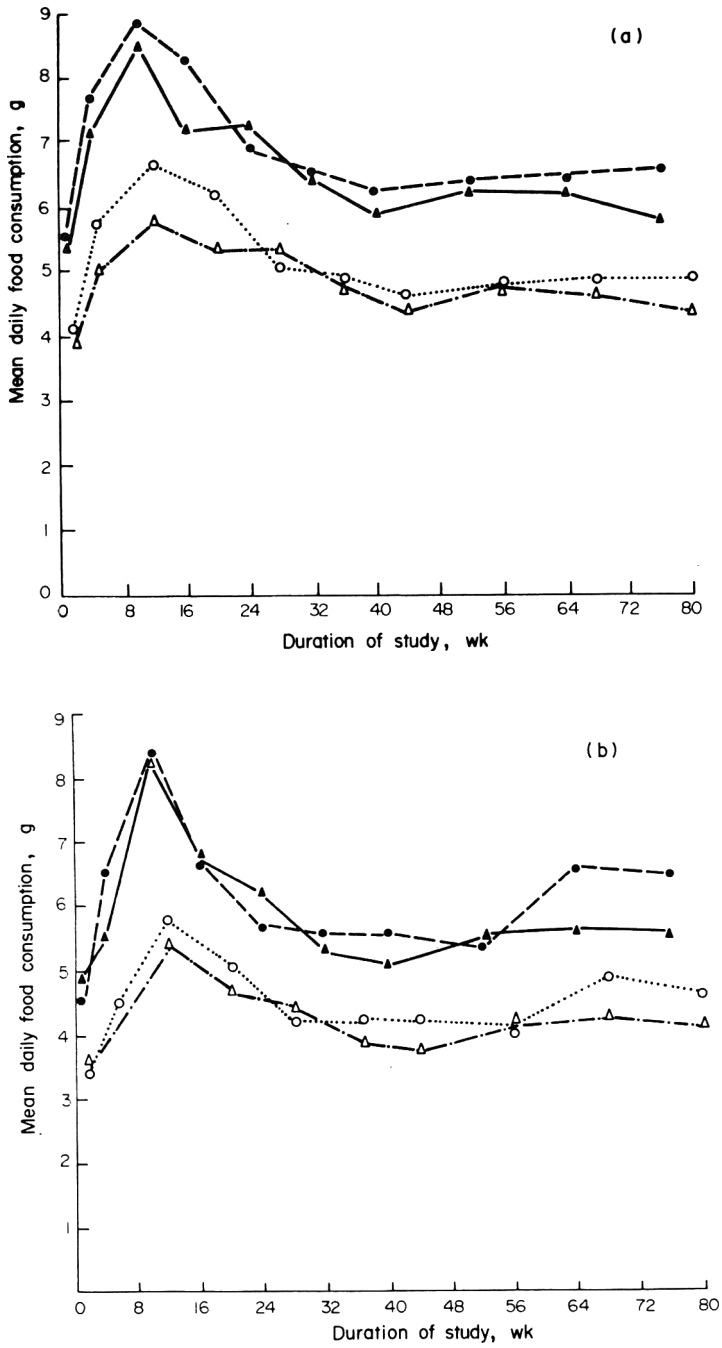

Fig. 1. Food consumption in (a) males and (b) females. Each point represents the mean for all living animals in each group. The groups were given: PRD diet *ad lib.* (-▲-), restricted PRD diet (···△···), 41B diet *ad lib.* (--●--) or restricted 41B diet (···○···).

($P < 0{\cdot}001$). In males the difference was reversed and not statistically significant.

Tumour incidence

For both diets and in both sexes dietary restriction significantly decreased the incidence of tumours at all sites (Table 3). Similarly significant differences were seen specifically in liver tumours in males while differences of lesser statistical significance were seen for lung tumours in both sexes.

Of the mice killed terminally, 25·7% had one or more neoplasms (Table 3). The incidence was far higher in groups fed *ad lib.* (37·5%) than in restricted groups (15·7%), this excess being significant in both sexes and on both kinds of diet. Malignant neoplasms were much rarer (5·5% total incidence) but again the incidence was significantly higher in groups fed *ad lib.* The excess tumour incidence in mice fed *ad lib.* was accounted for by an excess of both lung tumours and liver tumours, although this latter excess was much

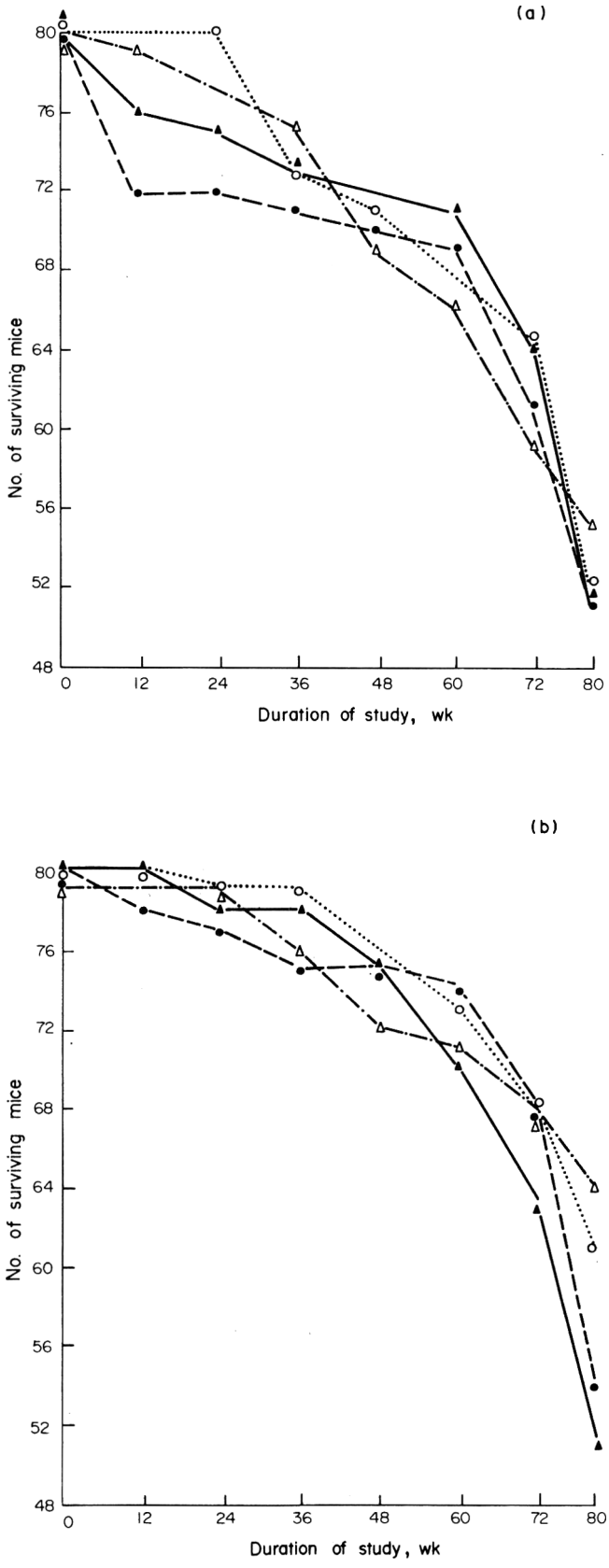

Fig. 2. Survival of (a) males and (b) females given: PRD diet *ad lib.* (-▲-), restricted PRD diet (·-△-·), 41B diet *ad lib.* (--●--) or restricted 41B diet (···○···).

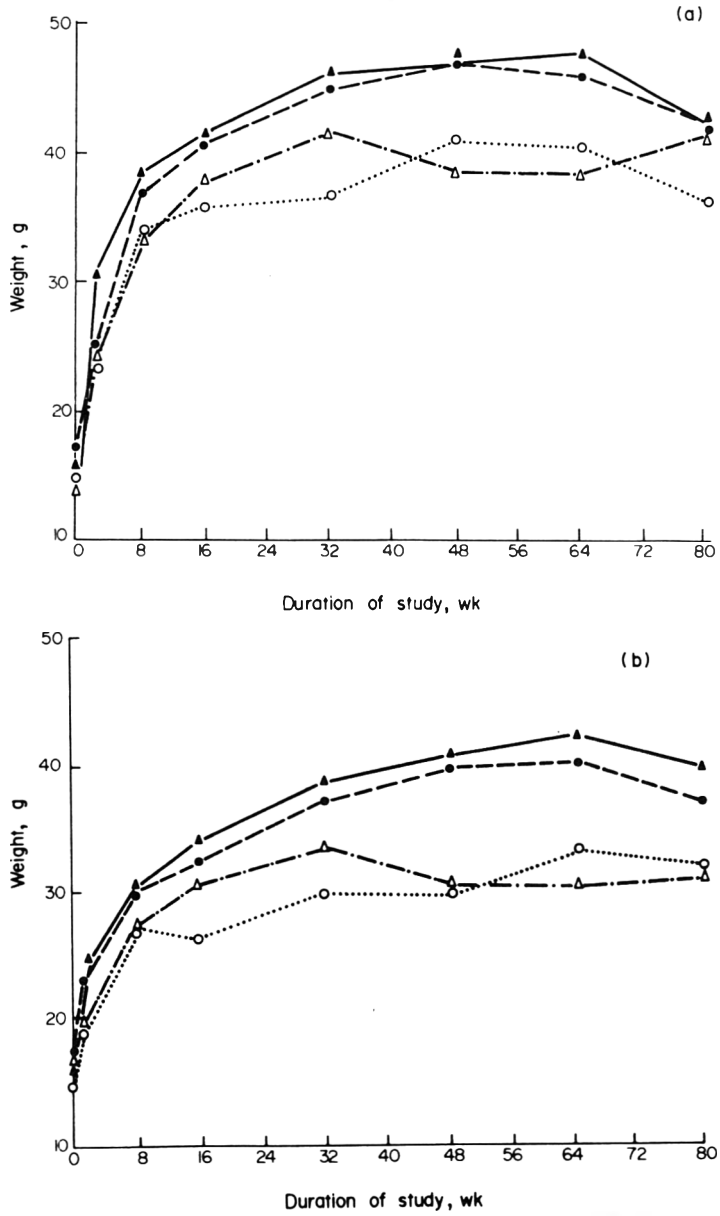

Fig. 3. Mean body weights of (a) males and (b) females given PRD diet *ad lib.* (–▲–), restricted PRD diet (·–△–·), 41B diet *ad lib.* (– –●– –) or restricted 41B diet (· · ·○· · ·).

Table 2. *Body and liver weights of mice with neither malignant lymphomas nor liver tumours that survived until the end of the study*

Dietary regime	No. of mice	Mean body weight (g)	Mean liver weight (g)	Relative liver weight (% of body weight)
		Males		
PRD *ad lib.*	37	44·1 ± 6·06	2·48 ± 0·54	5·6 ± 0·9
PRD restricted	51	41·1 ± 5·52	2·22 ± 0·36	5·4 ± 0·9
41B *ad lib.*	24	43·2 ± 5·66	2·39 ± 0·37	5·6 ± 0·8
41B restricted	44	36·2 ± 5·52	1·97 ± 0·42	5·4 ± 0·7
		Females		
PRD *ad lib.*	46	39·1 ± 6·44	1·98 ± 0·39	5·1 ± 0·8
PRD restricted	62	30·8 ± 4·84	1·78 ± 0·40	5·8 ± 1·0
41B *ad lib.*	47	37·1 ± 7·42	1·89 ± 0·45	5·1 ± 0·8
41B restricted	59	31·2 ± 3·8	1·67 ± 0·29	5·4 ± 0·9

Values are means ± SD.

Table 3. *Incidence of neoplasms in mice killed at end of test*

Dietary regime (sex)	No. of mice killed at end of test‡	No. of mice with ... Neoplasms at any site	Malignant neoplasms at any site	Lung tumours	Multiple lung tumours	Lung tumour of grade 2 or more§	Lung tumour of grade 3 or more§	Liver tumours	Multiple liver tumours	Liver tumour grade B, C or D‖	Liver tumour grade C or D‖	Neoplasms at sites other than lung or liver
PRD *ad lib.* (M)	48	19***	4	10	2	7	3	11***	4**	7*	0	3
PRD restricted (M)	55	8	1	6	0	4	1	2	0	2	0	1
41B *ad lib.* (M)	45	28***	4	13	1	10*	2	21****	7***	8	1	2
41B restricted (M)	51	16	1	10	3	5	0	6	0	5	0	1
PRD *ad lib.* (F)	48	12***	3	7**	0	5	0	2	0	0	0	4*
PRD restricted (F)	64	4	1	2	0	2	0	1	0	1	0	1
41B *ad lib.* (F)	51	14*	6	9	0	4	2	3*	0	2	1	5
41B restricted (F)	59	8	3	5	0	1	0	0	0	0	0	3
PRD *ad lib.* (M & F)	96	31****	7**	17**	2	12*	3	13***	4**	7	0	7**
PRD restricted (M & F)	119	12	2	8	0	6	1	3	0	3	0	2
41B *ad lib.* (M & F)	96	42****	10*	22*	1	14**	2	24****	7***	10	2	7
41B restricted (M & F)	110	24	4	15	3	6	0	6	0	5	0	4
PRD/41B *ad lib.* (M)	93	47****	8**	23	3	17**	5	32****	11****	15**	1	5
PRD/41B restricted (M)	106	24	2	16	3	9	1	8	0	7	0	2
PRD/41B *ad lib.* (F)	99	26***	9*	16**	0	9**	2	5*	0	2	1	9*
PRD/41B restricted (F)	123	12	4	7	0	3	0	1	0	1	0	4
PRD/41B *ad lib.* (M & F)	192	73****	17***	39***	3	26***	7	37****	11****	17**	2	14**
PRD/41B restricted (M & F)	229	36	6	23	3	12	1	9	0	8	0	6
PRD *ad lib.*/restricted (M & F)	215	43†††	9†	25	2	18	4	16†††	4	10	0	9
41B *ad lib.*/restricted (M & F)	206	66	14	37	4	20	4	30	7	15	2	11

M = Male F = Female

‡A total of 19 mice that died between weeks 80 and 83 have been excluded.

§Lung tumours were graded as follows (based on Walters, 1966): 1 = benign non-invasive adenoma; 2 = adenoma (extending into airways and/or into surrounding lung); 3 = adenocarcinoma with metastases in lobe of origin or entirely replacing one lobe; 4 = adenocarcinoma extending through pleura or metastasizing to lobes other than the lobe of origin; 5 = adenocarcinoma metastasizing to sites outside the thorax.

‖Liver tumours were graded as follows: A = consists of almost normal-looking liver cells in almost normal arrangement; B = consists of recognizable parenchymal cells arranged in cords; C = undoubtedly malignant liver cell tumour that has not metastasized elsewhere; D = metastasizing liver cell tumour.

Values marked with asterisks are significantly higher than the corresponding values given in the line below (*$P < 0.1$; **$P < 0.05$; ***$P < 0.01$; ****$P < 0.001$); those marked with daggers are significantly lower than the corresponding values in the line below (†$P < 0.1$; †††$P < 0.01$).

Table 4. *Incidence of lung and liver tumours in mice in relation to time of death*

Dietary regime (sex) Week no. ...	Cumulative no. of dead mice*...						
	1–29	30–59	60–69	70–74	75–79	80–83	0–83
PRD *ad lib.* (M)							
Total	3	4	6	5	7	52	77
With lung tumour	0	0	0	1	1	11	13
With liver tumour	0	0	1	1	2	13	17
PRD restricted (M)							
Total	4	9	4	2	5	55	79
With lung tumour	0	0	1	0	0	6	7
With liver tumour	0	0	1	0	0	3	4
41B *ad lib.* (M)							
Total	8	2	8	3	5	51	77
With lung tumour	0	0	1	2	1	13	17
With liver tumour	0	1	1	1	3	24	30
41B restricted (M)							
Total	2	10	4	2	10	52	80
With lung tumour	0	1	0	0	1	10	12
With liver tumour	0	0	0	1	1	6	8
PRD *ad lib.* (F)							
Total	2	5	6	4	10	51	78
With lung tumour	0	0	0	1	1	8	10
With liver tumour	0	1	0	0	1	2	4
PRD restricted (F)							
Total	2	6	2	2	3	64	79
With lung tumour	0	1	0	0	0	2	3
With liver tumour	0	0	0	0	0	1	1
41B *ad lib.* (F)							
Total	5	1	4	6	8	54	78
With lung tumour	0	0	1	2	2	9	14
With liver tumour	0	0	0	0	0	3	3
41B restricted (F)							
Total	1	5	6	2	5	61	80
With lung tumour	0	0	0	0	0	5	5
With liver tumour	0	0	0	0	0	0	0
All groups							
Total	27	42	40	26	53	440	628
With lung tumour	0	2	3	6	6	64	81
With liver tumour	0	2	3	3	7	52	67

M = Male F = Female

*Animals that did not receive a *post mortem* examination have been excluded. Animals that did not die sooner were killed from weeks 80 to 83.

more significant in male mice, which had a far higher liver-tumour rate than female mice. Tumours at sites other than lung or liver were not common (4·8% total incidence) and there was again an excess incidence in mice fed *ad lib.* and it was statistically significant for the two kinds of diet combined. The excess was also significant for mice on the PRD diet. Tumour incidences were significantly decreased in mice fed the PRD diet both for liver tumours alone and for all tumours combined. A higher incidence of liver and lung tumours in mice fed *ad lib.* was also indicated by considering the grades of these tumours and the number of animals that had more than one tumour. The lung and liver tumours observed in mice that died before the end of the study (Tables 4 and 5) only served to emphasize the difference between the two dietary levels.

Ophthalmoscopy

Routine ophthalmoscopy showed that the mice suffered from a high incidence (17%) of cataracts. This high incidence was also found in our breeding colony at Kimpton, and it was shown experimentally that the cataracts were a congenital abnormality of which the incidence was not influenced by the different diets or feeding regimes.

Amyloidosis of the kidney

Amyloidosis affected 78% of all animals surviving for 80 weeks. No relationship was found between incidence or severity of amyloidosis for either types of diet or feeding regime (Table 6).

Testicular changes

In male mice fed the 41B diet, but not in those fed PRD-diet testicular changes were significantly more frequent in mice fed restricted diets than in those fed *ad lib.* (see Table 7). Atrophy of the seminiferous tubules affected 66% of all male mice killed at the end of the test.

DISCUSSION

In the case of the two commercially available diets, mice fed on a restricted regime were smaller, sleeker, more active and had a slightly better survival rate up to 18 months than did mice fed *ad lib.* The diet-

Table 5. *Total incidence of lung and liver tumours in mice*

Dietary regime (sex)	Total no. of mice‡	No. of mice with... Lung tumour	Liver tumour
PRD *ad lib.* (M)	77	13	17***
PRD restricted (M)	79	7	4
41B *ad lib.* (M)	77	17	30****
41B restricted (M)	80	12	8
PRD *ad lib.* (F)	78	10**	4
PRD restricted (F)	79	3	1
41B *ad lib.* (F)	78	14**	3
41B restricted (F)	80	5	0
PRD *ad lib.* (M & F)	155	23**	21***
PRD restricted (M & F)	158	10	5
41B *ad lib.* (M & F)	155	31**	33****
41B restricted (M & F)	160	17	8
PRD/41B *ad lib.* (M)	154	30*	47****
PRD/41B restricted (M)	159	19	12
PRD/41B *ad lib.* (F)	156	24***	7**
PRD/41B restricted (F)	159	8	1
PRD/41B *ad lib.* (M & F)	310	54****	54****
PRD/41B restricted (M & F)	318	27	13
PRD *ad lib.*/restricted (M & F)	313	33†	26†††
41B *ad lib.*/restricted (M & F)	315	48	41

M = Male F = Female

‡Animals that did not receive a *post mortem* examination have been excluded.

Values marked with asterisks are significantly higher than the corresponding values given in the line below (*$P < 0{\cdot}1$; **$P < 0{\cdot}05$; ***$P < 0{\cdot}01$; ****$P < 0{\cdot}001$); those marked with daggers are significantly lower than the corresponding values in the line below (†$P < 0{\cdot}1$; †††$P < 0{\cdot}01$).

restricted mice developed fewer neoplasms during the 18-month period of observation.

Studies of the effect of dietary restriction on longevity and tumour incidence have been reported previously. Tannenbaum (1940, 1942, 1944 & 1945), Tannenbaum & Silverstone (1949 & 1950) and Silverstone & Tannenbaum (1951), carried out a series of classic nutritional and dietary studies. Restricting diet by reducing food quality (e.g. low calorific value, low protein, low fat) or quantity decreased the incidences of virus-induced mammary tumours in dba and C_3H mice, and of spontaneous lung tumours in Swiss mice. Similar results were obtained in rats where dietary restriction was associated with greatly increased life-span and lower tumour incidence.

Walters & Roe (1964) using mice injected on the first day of life with a chemical carcinogen, saw fewer lung tumours when the mice were subsequently fed on

Table 6. *Severity of amyloidosis of kidneys in mice killed at the end of the test*

Dietary regime (sex)	No. of mice killed‡	No. of mice with lesions of grades§... 0	1–5	2–5	3–5	4–5	5
PRD *ad lib.* (M)	48	14	34	27	15	7	2
PRD restricted (M)	55	16	39	26	13	6	0
41B *ad lib.* (M)	45	16	29	25	16	6	0
41B restricted (M)	51	8	43	34	23	10	3
PRD *ad lib.* (F)	48	18	30	26	17	8	0††
PRD restricted (F)	64	21	43	31	21	14	6
41B *ad lib.* (F)	51	16	35	30**	21	14	4
41B restricted (F)	59	23	36	21	17	11	6

M = Male F = Female

‡Nineteen mice that died between 80 and 83 wk have been excluded.

§Amyloidosis was graded as follows: 1 = minimal enlargement of glomerular tufts, all nuclei present; 2 = slight enlargement of glomerular tufts, most nuclei present; 3 = moderate enlargement of glomerular tufts, moderate number of nuclei present; 4 = moderate enlargement of glomerular tufts but only a few nuclei present; 5 = moderate enlargement of glomerular tufts, no nuclei present.

The value marked with asterisks is significantly higher (**$P < 0{\cdot}05$) than that for the corresponding group on the restricted diet; the value marked with daggers is significantly lower (††$P < 0{\cdot}05$) than that for the corresponding group on the restricted diet.

Table 7. *Testicular changes in mice killed at the end of the test*

Dietary regime	No. of mice killed*	Incidence of atrophy of the seminiferous tubules of grade‡.... 0	1–5	2–5	3–5	4–5	5	No. of mice with Leydig cell tumours
PRD *ad lib.*	48	31	17	16	10	3	0	1
PRD restricted	55	30	25	19	14	6	0	0
41B *ad lib.*	45	36	9††††	8†††	7††	5††	2	0
41B restricted	51	23	28	22	20	17	2	0

*Eleven males that died between 80 and 83 wk have been excluded.

‡Atrophy was graded as follows: 0 = one; 1 = occasional tubules showing slight atrophy; 2 = intermediate between grades 1 and 3; 3 = moderate numbers of tubules are atrophic; 4 = extensive atrophy of seminiferous tubules; 5 = complete atrophy.

Values marked with daggers are significantly lower (††$P < 0.05$; †††$P < 0.01$; ††††$P < 0.001$) than those of the corresponding groups on restricted diets.

a low protein diet than when they were fed on a high-protein diet. Rowlatt, Franks & Sheriff (1973) using C_3H/A^{vy} mice, a strain with a high incidence of spontaneous mammary and liver tumours reduced the tumour incidence and increased the life-span by offering only 1·5g of diet/day/mouse. Gellatly (1975), by increasing the amount of groundnut oil in the diet, found that C57BL mice killed after 80 wk had developed hepatic nodules proportional in number, size and activity to the amount of oil in the diet. Roe & Tucker (1973) found that dietary restriction was associated with significantly reduced incidence of malignant lymphoma, liver and lung tumours in mice and later Tucker (1979) confirmed that finding in mice and showed that in rats dietary restriction improved survival for up to 24 months and greatly reduced the incidence of mammary, pituitary and other neoplasms. McCay, Ellis, Barnes, Smith & Sperling (1939) reported an increase in average life-span and, on limited evidence, a lower incidence of tumours in weight-retarded rats. Berg & Simms (1960) observed delay in the onset of five types of lesions in rats restricted to 46% or 33% of their food intake compared with rats fed *ad lib.* Widdowson & Kennedy (1962), by restricting food intake during suckling, produced smaller rats that did not live longer but had a reduced tumour incidence. Ross & Bras (1965) found that lowering the protein, carbohydrate and total calories in the diet of rats, resulted in greater longevity and reduced tumour incidence. Nolen (1972) attempted to find a suitable rat model for long-term carcinogenicity studies using an enriched, well-balanced diet. The test group received 80% of the intake of the *ad lib.* group, resulting in smaller, healthier, longer-lived animals, with drastically reduced tumour incidence in males.

F. J. C. Roe (personal communication, 1978) has pointed out that the effects of dietary restriction are not necessarily solely attributable to reduced intake of calories. Animals on restricted dietary regimes tend to eat their ration soon after it is provided and, in any case are faced with an empty food hopper for a part of each 24 hours. In such animals, it is to be expected that blood corticosteroid levels will rise after they have consumed all the ration available to them, since this happens under conditions of starvation. It may be that a hormonal change of this nature occurring regularly every day, rather than mere reduction in calorie intake, is responsible for some of the profound differences between animals fed restricted diets and those fed *ad lib.* Another factor that may be important is that animals which feed continuously tend to have viable bacteria higher up in their gastro-intestinal tracts than animals feeding intermittently. Dietary restriction may thus result in changes in the distribution and nature of the gut flora and this might be involved in the beneficial effects of dietary restriction. Dr. Roe has also pointed out that *ad lib.* feeding cannot be regarded as 'natural'. Animals in the wild (or in zoos) would not normally enjoy a perpetual supply of food in excess of their daily requirements. It is really only an unfortunate custom that laboratory animals are normally fed *ad lib.*—a custom based partly on the desire to avoid the possibility of cruelty associated with starvation and partly on convenience in that animals fed *ad lib.* do not have to be provided with food every day.

The diets used in this present study were commercially available pelleted diets used by many research and breeding establishments. There was no difference in survival rates between the mice fed the two diets on either the *ad lib.* or the restricted regimes. The mice ate more of the 41B (maintenance) diet, than of the PRD (breeding) diet. The mice fed 41B diet had a higher total number of tumours and a significantly higher number of liver tumours than those fed PRD diet. This higher incidence of tumours, particularly liver tumours was associated with higher body weight, and may have been due to a higher intake of calories (Silverstone & Tannenbaum, 1949). Amyloidosis of the kidney appeared in a high percentage of all animals in all groups. Dietary manipulation appeared to have no significant effect on this disease, in this strain of mice. (Amyloid consists of a glycoprotein made up of a protein, probably globulin, combined with a sulphated polysaccharide. Unlike fat or glycogen, amyloid collects between rather than within the cells and fibres. In the kidney microscopic changes appear in the connective tissue of the vessels of the glomerular tufts, involving the walls of the arterioles and the connective tissue under the basement membrane of the collecting tubules. The glomeruli become converted into masses of amyloid through which no blood can pass). The high incidence and severity of amyloidosis encountered in the present study was associated with marked shortening of life-span due to renal failure. The relative contributions of genetic and environmental factors to the causation of amyloidosis are not known. In any case, however, the brevity of the life-

span would render mice of the stock used unsuitable for routine carcinogenicity testing.

The testicular change in the restricted 41B-fed mice was probably due to a marginal deficiency of vitamin E. Roe & Tucker (1974) speculated that atrophy of the seminiferous tubules in rats fed a restricted diet, was due to reduced vitamin E availability. The 41B diet used in this experiment had a lower vitamin E content than the PRD (39 IU/kg diet compared with 48 IU/kg diet: see Table 1). L.A.S.A. (1969) recommend a diet containing a level of 55 IU/kg (60 mg/kg) of vitamin E.

The strain of mice used suffered from tumours common to most outbred strains of mice, i.e. lung tumours, liver-cell tumours and lymphoreticular tumours whereas mammary and Leydig-cell tumours were rare. Although tumour incidence in these mice was perhaps acceptable for the use of the strain in long-term tests, the high incidences of cataract and and atrophy of the seminiferous tubules and the very high incidence of amyloidosis of the kidney render this strain unsuitable for such tests.

Of the two diets used in this test PRD diet appears to be marginally better for long-term studies. Although it costs more, and has a higher protein level, less is eaten and there is a slightly lower incidence of tumours up to 18 months. The advantages of conducting long-term carcinogenicity tests under conditions of dietary restriction include those listed below.

(i) A substance that depressed appetite under conditions of *ad lib.* feeding and therefore lowered tumour incidence might prevent the detection of a carcinogen.

(ii) A substance that increased appetite under conditions of *ad lib.* feeding and therefore increased tumour incidence might cause a non-carcinogen to be branded as a carcinogen.

(iii) The high background incidence of 'spontaneous' tumours in animals fed *ad lib.* would make evaluation of carcinogenicity difficult.

(iv) The increased survival of animals on restricted diets allows a longer period of exposure to the test compound.

(v) Animals fed on restricted regimes, being smaller, would require less of the test agent to achieve the same dose on a unit/body weight basis.

(vi) Restricted mice tend to eat all the food offered. Consequently, during feeding experiments, food consumption can be estimated easily and accurately because wastage due to spillage is negligible and consumption of the test agent can be accurately calculated.

(vii) Less diet is used, cutting the cost of food.

These factors have led us to the view that the smaller, sleeker animal with a lower tumour incidence produced by restricted dietary intake is a more suitable model for carcinogenicity studies than an obese animal fed *ad lib.*

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SHORT PAPERS

N-NITROSODIMETHYLAMINE IN HUMAN BLOOD

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Abstract—Whole blood from 38 healthy persons (19 males and 19 females) was analysed for volatile nitrosamines. *N*-Nitrosodimethylamine was detected in blood samples from 97% of the individuals tested at a mean value (± 1 SD) of 0·6 ± 0·4 ng/ml.

Introduction

Volatile nitrosamines have been reported in the urine (Brooks, Cherry, Thacker & Alley, 1972; Hicks, Gough & Walters, 1978; Radomski, Greenwald, Hearn, Block & Woods, 1977) and faeces (Wang, Kakizoe, Dion, Furrer, Varghese & Bruce, 1978) of healthy individuals, in gastric contents from patients with gastro-intestinal disorders (Lakritz, Wasserman, Gates & Spinelli, 1978), and in the blood from one individual (Fine, Ross, Rounbehler, Silvergleid & Song, 1977). These findings suggest that *in vivo* nitrosation may occur in man. Because of the potent carcinogenic properties of nitrosamines, as shown by animal studies (Magee & Barnes, 1967), it would be important to demonstrate the presence of dialkylnitrosamines in the blood. This paper reports on the evidence for volatile nitrosamines obtained from examination of blood from normal men and women and on the detection of *N*-nitrosodimethylamine (NDMA) in 97% of the individuals tested.

Experimental†

Blood sampling. Blood samples from 38 normal subjects (19 men and 19 women, aged 21–60 yr; mean age 38 yr) were analysed for volatile nitrosamines. A syringe was used to collect all blood samples, which were immediately transferred to glass beakers and made basic. Commercially available evacuated blood-collection tubes were not used to collect the blood samples because we found that some rubber stoppers used to seal these tubes contained volatile nitrosamines (Lakritz & Kimoto, 1980). Additional fasting blood samples from one individual were collected periodically over a 6-month period to ascertain any variation in nitrosamine content.

Extraction procedure. Prior to initiating the study, the extraction of volatile nitrosamines in whole blood by distillation from alkali was compared with the use of a column-chromatographic technique using Na_2SO_4 and Celite without the application of heat. Our results indicated that both procedures were quantitatively comparable and that neither induced the formation of artefacts.

Analysis. In preliminary studies, we found that NDMA and the internal standard (*N*-nitrosomethylethylamine) were equally distributed in the cellular elements of the blood and in the plasma or serum. Therefore, it was advantageous to analyse whole blood samples, permitting work on samples as small as 10 ml. The volatile nitrosamines were analysed by a modification of a procedure described by Telling, Bryce & Althorpe (1971), involving distillation of whole blood from an alkaline solution. The distillate was collected and extracted with methylene chloride and the extracts were concentrated by evaporation. Analyses were performed with a gas–liquid chromatograph (GC) interfaced with a Thermal Energy Analyzer (TEA) Model 502 (Waltham, MA), a selective nitrosamine detector, operated under conditions similar to those employed by Fine & Rounbehler (1975). The levels of NDMA were determined by comparison with known concentrations of NDMA found to be linear over a range 0·1–10·0 ng/ml; the minimum confidence level of detection was 0·1 ng/ml. Where apparent nitrosamines were identified on the basis of GC retention time and TEA detectability, further confirmation was obtained by ultraviolet photolysis (Doerr & Fiddler, 1977).

Several preliminary experiments were conducted to ensure the accurate determination of concentrations of detectable nitrosamines in blood. Neither changes

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†Note: Precaution should be exercised in the handling of nitrosamines since they are potential carcinogens.

Table 1. *N-Nitrosodimethylamine in whole blood from healthy subjects*

Subjects	No. tested	*N*-Nitrosodimethylamine (ng/ml)* Range	Mean ± 1 SD
Total	38	0–1·5	0·6 ± 0·4
Male	19	0·2–0·8	0·4 ± 0·2
Female	19	0–1·5	0·8 ± 0·4
Fasted	25	0–1·5	0·6 ± 0·4
Non-fasted	13	0·2–1·2	0·5 ± 0·3
Smokers	11	0–1·2	0·7 ± 0·3
Non-smokers	27	0·2–1·2	0·5 ± 0·3

*ng/ml ≅ ppb ≃ μg/kg.

in the concentration of nitrosamines nor formation of artefacts were observed when 3 N-KOH or heparin was added to freshly drawn blood and the blood was stored in a frozen state for up to 3 days. All reagents and sample blanks used in this study were checked and found to be free from TEA-responsive peaks. Degassed deionized distilled water was used throughout the analyses. The errors associated with the whole-blood samples sizes of 10, 20 and 100 ml were found to be 0·15, 0·10 and 0·10 ng NDMA/ml, respectively. Ten determinations performed on the same blood specimen by three different analysts showed the NDMA content to be 0·3 ± 0·1 ng/ml (mean ± 1 SD). An internal standard, *N*-nitrosomethylethylamine, was added to each blood sample to ensure the reliability of each assay. Recoveries of the internal standard added at 1·0 and 2·0 ng/ml were 94 ± 5%. For statistical purposes, the blood-nitrosamine data were grouped according to sex, to whether samples were taken under fasting or non-fasting conditions and to whether the donors were smokers or non-smokers, and were subjected to analysis of variance.

Results

When blood samples from 38 subjects were assayed for volatile nitrosamines, 37 were found to contain NDMA. The results are shown in Table 1. The whole-blood NDMA level in these assays was 0·6 ± 0·4 ng/ml. Blood from one of the non-fasting males also contained 1·6 ng *N*-nitrosodiethylamine/ml. A higher concentration of NDMA appeared to be present in the blood of females (0·8 ± 0·4 ng/ml) than of males (0·4 ± 0·2 ng/ml). However, when fasting females were compared to fasting males no significant difference was observed. Neither were significant differences in the blood NDMA levels noted when smokers were compared to non-smokers. Fasting blood drawn periodically from one subject over a 6-month period showed a mean value of 0·5 ± 0·2 ng/ml.

Discussion

Volatile nitrosamines have been shown to be potent carcinogens when tested in a number of laboratory animals. Although no case of human cancer has been attributed to these compounds, recent reports have indicated that they are present in the urine (Brooks *et al.* 1972; Hicks *et al.* 1978; Radomski *et al.* 1977), in faeces (Wang *et al.* 1978), and in gastric contents (Lakritz *et al.* 1978). It is important, therefore, to determine whether nitrosamines are present in blood, whether they are of exogenous or endogenous origin (or both) and whether they are carcinogenic to man.

The data in this report addresses the first of these questions, and indicates that over 95% of the healthy individuals sampled for this study had measurable levels of NDMA in their blood.

The method used has a sensitivity of 0·1 ng/ml with a 95% recovery, so that the mean level of 0·6 ng/ml can be regarded as a valid result. These findings are comparable to results by Fine *et al.* (1977), who reported concentrations of 0·35 ng NDMA/ml in blood from a single subject before a meal of spinach, bacon, tomato, bread and beer, and 0·77 ng/ml 35 min later.

Nitrosamines in the blood may originate from ingestion of food containing preformed nitrosamines, from inhalation or from *in vivo* formation. Magee & Faber (1962) postulated a metabolic sequence for NDMA, as with other dialkylnitrosamines, which are also pre-carcinogens, with direct carcinogenic activity occurring only after activation through a series of demethylation and oxidative steps. The liver is probably the main metabolic site for this activation, and the presence of NDMA in human blood raises the question of the nitrosamine load and the rate of metabolic activation. The relationship of the findings reported in this study to the pathogenesis of cancer in man requires further investigation.

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LACK OF CARCINOGENIC EFFECT OF NITROSOCHLORDIAZEPOXIDE AND OF NITROSOMETHYLPHENIDATE GIVEN ORALLY TO MICE

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Abstract—The nitroso derivatives of two widely used psychotropic drugs, chlordiazepoxide and methylphenidate, were each administered in the drinking-water of (C57BL/6 × BALB/c) F_1 mice at concentrations of 50 and 100 mg/litre, respectively. The mice were treated on the first 4 days of each week from weaning until they were 18 months old. They were autopsied when moribund or at 26 months of age. No increase in tumour incidence, compared to controls, occurred as a result of treatment with either nitrosamine. The mice exposed to nitrosochlordiazepoxide had a significantly lower overall incidence of spontaneous tumours than controls.

Introduction

A variety of pharmaceutical agents that are secondary or tertiary amines could interact with nitrite in the stomach (Lijinsky, 1974; Lijinsky, Conrad & Van de Bogart, 1972; Serfontein & de Villiers, 1975) or in saliva (Rao, 1978). It is, therefore, of interest to determine whether such nitrosamine derivatives are carcinogenic in animals and, thus, of potential risk to man. We have tested the *N*-nitroso derivatives of two commonly used psychotropic drugs, chlordiazepoxide and methylphenidate, by long-term (17 months) administration in the drinking-water of mice followed by lifetime observation.

Experimental

Chemicals. Nitrosomethylphenidate was prepared from methylphenidate hydrochloride kindly supplied by Ciba-Geigy, Inc., Summit, NJ. The drug (36·0 g; 0·133 mol) was dissolved in 15% aqueous acetic acid (250 ml), sodium nitrite (38·0 g; 0·55 mol) in 30% aqueous solution was added slowly at 5°C and the mixture was stirred for 3 hr. The resulting precipitate was collected, thoroughly washed with cold water and dried *in vacuo* (34·8 g; 98% yield; m.p. 102–104°C). Elemental analysis, thin-layer chromatography (TLC) and ultraviolet spectroscopy indicated the identity and purity of the product. A similar preparative method has been described (Lijinsky & Taylor, 1976).

Nitrosochlordiazepoxide was prepared similarly, from chlordiazepoxide hydrochloride kindly supplied by Hoffmann-LaRoche, Inc., Nutley, NJ. The drug (15 g; 0·045 mol) was dissolved in 0·1 N-HCl (300 ml), sodium nitrite (0·182 mol) in 30% aqueous solution was added slowly at 5°C and the mixture was stirred for 3 hr. The resulting precipitate was collected, thoroughly washed with cold water and dried *in vacuo* (14·7 g, a quantitative yield; m.p. 152–154°C). Elemental analysis, TLC and ultraviolet spectroscopy indicated the identity and purity of the product. A similar method of preparation has been reported (Walser, Fryer, Sternbach & Archer, 1974).

Animals and treatment. C57BL/6 females and BALB/c males, obtained from the Jackson Laboratories, Bar Harbor, Maine, were used to obtain F_1 hybrids. This hybrid has been found to be a long-lived disease-resistant model for carcinogenesis assays. The mice were housed in clear plastics cages with filter tops and were supplied with hardwood shavings as bedding. They were fed Purina Mouse Chow (Ralston-Purina Co. Inc., St. Louis, MO). The room was maintained at 25 ± 2°C and 40–60% humidity, and had a fluorescent light/dark cycle of 14/10 hr.

Drinking-water solutions of *N*-nitrosomethylphenidate (100 mg/litre) were prepared by dilution of a 25 mg/ml ethanolic stock solution. *N*-Nitrosochlordiazepoxide (50 mg) was dissolved in ethanol by heating at 45°C for a few minutes; this solution was added to the drinking-water to give a concentration of 50 mg/litre. The solutions were protected from light with aluminium foil and were found to be stable under these conditions. Each cage of five mice received 100 ml of the appropriate drinking solution per week. When the entire volume had been consumed (over about 4 days) the mice received tap-water for the remainder of the week. Treatment was begun when the mice were 4 wk old and was continued until they were 18 months old. The total dose received per mouse was approximately 150 mg for nitrosomethylphenidate and 75 mg for nitrosochlordiazepoxide. The estimated daily doses were

Table 1. *Effect of prolonged administration of nitrosochlordiazepoxide or nitrosomethylphenidate in drinking-water on tumour incidence in mice*

			Incidence of					
			Primary lung tumours		Other neoplasms		All tumours	
Treatment†	Mice/group	Mean age at death (months)	No. of mice with tumour‡	Mean no. of tumours/ tumour-bearing mouse	No. of mice with tumour‡	Type	No. of mice with any tumour‡	No. of mice with lung tumour(s) plus other types of tumour‡
Control	18M	21·7 ± 3·5	15 (83)	1·6 ± 0·6	8 (44)	3 hepatic tumours 3 lymphomas 3 sc sarcomas	16 (89)	7 (39)
	43F	24·6 ± 2·2	14 (33)	1·2 ± 0·6	13 (3)	11 lymphomas 2 sc sarcomas	24 (56)	3 (7)
NCDE	23M	25·3 ± 2·6	14 (61)	1·4 ± 0·6	2 (9)	1 hepatic tumour 1 lymphoma	15 (65)	1 (4)*
	21F	25·4 ± 2·4	6 (29)	1·2 ± 0·4	1 (5)	1 lymphoma	6 (29)*	1 (5)
NMPh	24M	22·2 ± 3·5	17 (71)	1·9 ± 1·3	6 (25)	3 hepatic tumours 2 lymphomas 1 splenic sarcoma 2 sc sarcomas	19 (79)	4 (17)
	31F	24·1 ± 2·8	7 (23)	1·3 ± 0·8	9 (29)	7 lymphomas 1 bone angiosarcoma 1 papillary eye tumour	15 (48)	1 (3)

†Mice were given NCDE (50 mg nitrosochlordiazepoxide/litre drinking-water) or NMPh (100 mg nitrosomethylphenidate/litre) from the age of 4 wk to 18 months and then held until moribund or 25–26 months old.

‡Each number in brackets is the preceding value expressed as a percentage of the total number of mice in the group. Means are expressed ± 1 SD. Values marked with an asterisk differ significantly from the control figure (*$P < 0.05$ by chi-square test).

12·5 mg/kg for nitrosomethylphenidate and 6·2 mg/kg for nitrosochlordiazepoxide. These doses are several orders of magnitude greater than the human exposure to be anticipated from *in vivo* nitrosation of methylphenidate (given in quantities up to 1 g/day) or of chlordiazepoxide (20 mg/day or less).

The mice were killed with CO_2 when moribund or at 25–26 months of age. All tumours and other lesions were fixed in Bouin's solution for histopathological examination (7-μm sections stained with haematoxylin and eosin). All alveologenic pulmonary neoplasms were classified as primary lung tumours. Hepatic neoplasms were classified according to Squire & Levitt (1975) and included neoplastic nodules and carcinomas. Statistical tests used were the chi-square test, with Yates' correction where indicated, for differences in percentages of tumour-bearing mice, and Student's *t* test using Poisson distribution for differences in the number of tumours per mouse.

Results and Discussion

No significant non-neoplastic lesions were discovered in treated or control mice. The most common neoplasms found in our mice were primary lung tumours; these occurred more frequently in males than in females (Table 1). Other neoplasms that were observed in more than one mouse were lymphomas, hepatic neoplastic nodules and carcinomas, and soft tissue (subcutaneous) sarcomas. Treatment with nitrosochlordiazepoxide or with nitrosomethylphenidate did not increase the incidence of any of these tumours or the total tumour incidence. In fact, the mice exposed to nitrosochlordiazepoxide experienced a significant reduction in tumour incidence, compared with the controls, with regard to the percentage of male and female mice bearing neoplasms other than lung tumours, the percentage of female mice bearing all types of tumour, and the percentage of male mice bearing one or more lung tumours plus one or more tumours of another type.

The percentage of mice bearing a lung tumour was large, especially among the males. A high spontaneous incidence of murine lung tumours invariably indicates high sensitivity of the lungs to induction of these tumours by carcinogens (Shimkin & Stoner, 1975). Since the multiplicity of the spontaneous lung tumours in our experiment was low (less than two), we could readily have detected a carcinogenic effect resulting in increased tumour multiplicity, a well-established carcinogen bioassay endpoint (Shimkin & Stoner, 1975).

Nitrosomethylphenidate has been reported to be non-carcinogenic in rats (Lijinsky & Taylor, 1976). Our results with mice confirm that this nitrosated drug is not a carcinogen. Lijinsky & Taylor (1977) observed neurogenic tumours in a low, statistically insignificant percentage of rats given chlordiazepoxide plus nitrite. The appearance of these rare tumours was considered to be suggestive but not conclusive evidence of a carcinogenic effect. In our experiment with mice, however, nitrosochlordiazepoxide was not only non-carcinogenic but even reduced the incidence of spontaneous neoplasms. Although unique sensitivity of the human species cannot be ruled out, we conclude that nitrosation products of chlordiazepoxide and methylphenidate probably do not contribute greatly to human cancer risk.

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TUMOURS INDUCED IN FISCHER 344 RATS BY THE FEEDING OF DISULFIRAM TOGETHER WITH SODIUM NITRITE

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Abstract—A mixture of 0·1% disulfiram together with 0·2% sodium nitrite in powdered rat diet was fed to 20 male and 20 female Fischer 344 rats for 78 wk, after which the animals were observed until death. Ten of the males and 12 of the females died with tumours of the oesophagus, tongue, squamous stomach and nasal cavity. None of these tumours was observed in rats fed either disulfiram or sodium nitrite alone at similar doses. The tumours were attributed to the reaction of disulfiram and nitrite in the stomach, with the formation of nitrosodiethylamine, which has given rise to these tumours in Fischer rats from the same colony.

Introduction

Following the discovery of the carcinogenicity of nitrosamines by Magee & Barnes (1956), the possibility was raised that exposure to these compounds could result from their formation in the stomach from ingested secondary amines and nitrite (Druckrey, Steinhoff, Beuthner, Schneider & Klärner, 1963). Although that first experiment using diethylamine and nitrite failed, presumably because the strong basicity of diethylamine led to a slow rate of reaction between these two compounds, the principle was not forgotten. The first positive experiment demonstrating formation of tumours by the feeding of a secondary amine and nitrite to animals was reported by Sanders & Bürkle (1969).

The wide significance of this possibility—the formation of nitrosamines *in vivo*—was discussed by Lijinsky & Epstein (1970). Later the possibilities became broader with the recognition that tertiary amines were other possible sources of nitrosamines through reaction with nitrite in the stomach (Lijinsky, Keefer, Conrad & Van de Bogart, 1972b; Lijinsky & Singer, 1974). Tertiary amines tended to react less readily with nitrous acid than do secondary amines (Jones, Lijinsky & Singer, 1974), with notable exceptions such as aminopyrine (Lijinsky & Greenblatt, 1972), but they could, nevertheless, represent a significant source of nitrosamines.

Several tertiary amines have been examined as possible sources of nitrosamines in the environment and in man. These include triethanolamine (Lijinsky *et al.* 1972b), several agricultural chemicals (Elespuru & Lijinsky, 1973) and drugs (Lijinsky, Conrad & Van de Bogart, 1972a). The drugs are of special interest because so many of them are tertiary amines (House of Representatives Hearings, 1971).

Most of the tertiary amines studied have given rise to detectable amounts of nitrosamines when reacted with nitrite in moderately acidic conditions similar to those prevailing in the mammalian stomach during digestion of a meal (Lijinsky *et al.* 1972a). Among these was disulfiram, which yielded nitrosodiethylamine (NDEA; Lijinsky, 1974). Several of the chemical tests were followed by long-term tests comprising administration of the tertiary amine together with nitrite to rats. Both the amine and sodium nitrite were dissolved in drinking-water, an easy procedure, but one that was not entirely satisfactory because of the small possibility that some slight reaction might have occurred in the aqueous solution itself. In these early experiments the feeding of the combinations of most of the amines with nitrite did not lead to induction of a significant incidence of tumours in the rats (Lijinsky & Taylor, 1977a,b), although a few of the combinations were carcinogenic.

Clearly, administration in drinking-water was not feasible for testing tertiary amines that were insoluble or only slightly soluble in water. Among these was disulfiram (bis(diethyldithiocarbamoyl) disulphide) used as a fungicide and rubber accelerator under the name Ethyl Tuads and as a drug under the name Antabuse for the treatment of alcoholics. Accordingly, the testing of this and similarly insoluble compounds was carried out by feeding a mixture in the powdered feed containing sodium nitrite. The treatment was continuous for 78 wk, after which the animals were observed until they died. All tumours and other lesions were compared with those in animals fed the disulfiram or sodium nitrite alone.

Experimental

Chemicals. Disulfiram was a chemically pure material of quality suitable for drug use and was kindly provided by Ayerst Laboratories, New York. The sodium nitrite was from Fisher Scientific Company, Pittsburgh, PA.

Animals and treatment. Groups of 20 male and 20 female Fischer 344 rats, bred and raised in the colony of the Frederick Cancer Research Center, were 8–9 wk old at the beginning of treatment. The animals were housed four to a polycarbonate cage on hard-

wood-chip bedding. Disulfiram (0·1%) and sodium nitrite (0·2%) were mixed with powdered Wayne Sterilizable Lab Meal (Allied Mills, Inc., Chicago, IL) in a blender. The mixing of the ingredients was uniform, as shown by chemical analysis of random samples of the mixture, after the blending had been continued for 5 min. This mixture was prepared at first every 2 wk and then weekly and was stored at 4°C until placed in the feeders every 2–3 days. Analysis of the food by extraction with ether followed by gas chromatography showed the presence of a trace of NDEA after 7 days storage and no more than 200 μg/kg after 17 days. The animals were supplied with the food containing the chemicals *ad lib.* for 78 wk, after which they were observed until natural death. On the basis of a food intake estimated as 30 g/day for male rats and 20 g/day for female rats, the males ingested 30 mg disulfiram and 60 mg sodium nitrite/day, and the females 20 mg disulfiram and 40 mg sodium nitrite/day. Surviving animals (one male and eight females) were killed at wk 117. All animals were given a complete autopsy, including examination of all major organs and tissues. These organs and tissues and all lesions were fixed for histopathological examination.

A control group of 20 male and 20 female rats was fed 0·2% sodium nitrite. No control group fed only disulfiram was maintained in this experiment because overlapping with this study was a standard NCI bioassay of disulfiram (Ethyl Tuads), which under the conditions of the study was not considered to be carcinogenic (NCI Carcinogenesis Technical Report Series, No. 166, 1979).

Results and Discussion

The pattern of mortality in the rats fed a mixture of disulfiram and sodium nitrite in the diet is shown in Table 1. More than half of the animals were alive 100 wk after the beginning of treatment, but of the 40 nitrite-fed control rats, 19 were still alive at wk 122, and in rats treated in another study with disulfiram alone the survival rate was almost 90% at wk 100 of treatment. The somewhat earlier occurrence of deaths in the group fed the disulfiram–nitrite mixture reflected a different pattern of tumour induction in this group.

Table 1. *Mortality of Fischer rats fed 0·1% disulfiram and 0·2% sodium nitrite in the diet for 78 wk*

	No. of survivors	
Wk no.	Males	Females
0*	20	20
50	20	20
60	20	19
70	20	19
80	18	17
90	17	16
100	12	11
110	3	9
117†	1	8

*Beginning of treatment.
†When all survivors were killed.

Table 2. *Tumours in Fischer rats fed 0·1% disulfiram and 0·2% sodium nitrite in the diet for up to 78 wk*

Location of tumours	No. of animals affected*	
	Males	Females
All sites	10	12
Oesophagus	7	11
Tongue	0	2
Squamous stomach	3	0
Nasal cavity	2	2

*None of the tumours found in these animals was observed in 50 male and 50 female rats given 0·06% disulfiram in the diet for 107 wk, nor in 20 male and 20 female rats given 0·2% sodium nitrite in the diet for 120 wk.

In Table 2 are listed the numbers of animals with several types of tumours of the upper gastro-intestinal tract and of the nasal cavity. The gastro-intestinal tumours were mainly basal-cell carcinomas and papillomas of the oesophagus, tongue and forestomach. The tumours of the nasal cavity were olfactory adenocarcinomas. These tumours have been fully described elsewhere (Lijinsky & Taylor, 1975). More than half of the animals treated with disulfiram and nitrite, both males and females, had tumours that are not seen in untreated Fischer rats of our colony (Goodman, Ward, Squire, Chu & Linhart, 1979). Tumours of the oesophagus were most common, accounting for the deaths of almost half of the treated animals. No such tumours were seen in the 50 male and 50 female rats treated with 0·06% disulfiram in the diet for 107 wk in the NCI bioassay, although the total dose in this case was very similar to that administered in the test of disulfiram with nitrite. Nor have any of these tumours been observed in the nitrite-treated controls or in previous tests on sodium nitrite conducted in several other laboratories. The tumours found in the 21 nitrite-fed controls that have died have been of the types, mainly of endocrine origin, found in untreated control rats of our colony.

The conclusion must be drawn that the feeding of the combination of disulfiram and nitrite gave rise to the tumours reported. The reaction of disulfiram with nitrite in mildly acid solution has been shown to yield NDEA (Lijinsky, 1974; Likinsky *et al.* 1972a) and it is reasonable to deduce that this nitrosamine would have been produced in the stomach of the rats during this experiment. We have recently concluded, but not yet published, tests of NDEA in Fischer rats, in which tumours have been observed in the oesophagus and squamous stomach after doses of 140 μg/rat/day administered in the drinking-water for 30 wk. Of the 20 treated animals, 15 were dead with tumours of the oesophagus by wk 70. In experiments reported by Druckrey, Schildbach, Schmähl, Preussmann & Ivankovic (1963), rats were given 0·075 mg NDEA/kg/day (20–40 μg/rat) and the incidence of tumours found after lifelong treatment was very low.

While the doses of NDEA received by the rats were small and it is not possible to estimate the effect in man of even smaller doses of this carcinogen, such as might arise through reaction of disulfiram with nitrite

in the human stomach, it seems clear that exposure of people to disulfiram might present some risk. If the compound is ingested either incidentally or as a drug, there is a probability that some reaction will take place with nitrite in the acid medium of the stomach, because nitrite either from saliva or in cured meats can be present in the stomach contents. Exposures to NDEA from this source, even though the quantities are small, might contribute to an increased carcinogenic risk if they occurred frequently over a span of many years.

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. An Encyclopedic Handbook. Volume 2, Mycotoxicoses of Domestic and Laboratory Animals, Poultry, and Aquatic Invertebrates and Vertebrates. Edited by T. D. Wyllie and L. G. Morehouse. Marcel Dekker, Inc., New York, 1978 pp. xxv + 570. Sw. fr. 230.00.

Toxicology, Biochemistry and Pathology of Mycotoxins. Edited by K. Uraguchi and M. Yamazaki. Kodansha Ltd., Tokyo, and John Wiley & Sons, New York, 1978. pp. viii + 288. £19.50.

Following an initial volume concerned with the identification of fungi that produce mycotoxins, the chemistry of the toxic metabolites and the methods for their extraction, isolation and identification, Volume 2 of the three volume handbook cited above embarks upon a detailed consideration of the effects of a wide range of mycotoxins in animals.

The authors of a general review of mycotoxicosis in a range of species including poultry point out that animals that fall ill as a result of eating food contaminated with fungi present the 'vet' with major problems in diagnosis. In a few cases, the toxins may be identified readily from the history of the outbreak and the clinical signs and pathological lesions, but in the rest the causative agent is far from obvious.

Subsequent contributions deal systematically with the mycotoxicoses of cattle, sheep, horses, swine and poultry, enabling the reader to obtain with ease an in-depth coverage of a particular speciality. The sections are cross-referenced to the causal fungi and to the specific toxins involved, and provide information on the pathology and clinical signs of each disease, as well as on its occurrence, control and treatment.

The next major section reviews mycotoxicoses in laboratory animals, including the cat, dog, guinea-pig, hamster, mouse, non-human primate, rabbit and rat. Most of the information presented is derived from experimental studies, since among experimental animals there have been few clearly defined cases of spontaneously occurring disease due to mycotoxins.

The final section in this volume deals with aquatic animals. Among the topics included are aflatoxin-induced hepatomas in rainbow trout, and the less notorious sensitivity of brine-shrimp larvae and zebra-fish larvae to many of the mycotoxins. The authors of this section reiterate the attributes that make the rainbow trout, which is highly sensitive to aflatoxin, a particularly suitable model for the study of liver carcinogens. These include a short latent period for tumour induction, drug-metabolizing enzyme systems similar to those in mammals, and low maintenance costs.

Like Volume 3, reviewed earlier (*Cited in F.C.T.* 1979, **17**, 537), this book is a most useful source of reference for the research scientist, veterinary surgeon and toxicologist. The text is well laid out and is supported by clear photographs, a comprehensive glossary and an author and subject index.

The second book named above offers a different approach to the mycotoxin problem, presenting in turn contributions on mycotoxin research as applied to food contamination, the chemistry of mycotoxins, their toxicology and biochemistry and the types of change induced by different mycotoxins in various cells and tissues. The penultimate chapter reviews the field of fungal carcinogens, and finally a contribution concerned solely with aflatoxin looks into its occurrence in traditional Japanese foods and imported products. The contributors are all from Japan, a country where mycotoxin research has been well established for many years, and their surveys are concerned particularly with the role of mycotoxins in human health.

Side Effects of Drugs Annual 3—1979 A Worldwide Yearly Survey of New Data and Trends. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1979. pp. xvi + 469. Dfl. 120.00.

This latest supplement to Volume VIII of *Meyler's Side Effects of Drugs* (*Cited in F.C.T.* **15**, 241) follows the general pattern of its two predecessors. It covers all reports that were published between 1 August 1977 and 1 July 1978 and were considered to present significant new data on adverse reactions to drugs. In addition, some more recent papers have been included. The information is again indexed separately under drugs, synonyms and side effects, with each index covering all three supplements to Volume VIII.

While most of the information presented relates specifically to drugs, other constituents of pharmaceutical preparations are included. Entries may be found, for example, on tartrazine and other colourings, polyethylene used in plastic surgery, cyclamate and other constituents of special diets, sunscreening agents and a variety of additives and vehicles, including propylene glycol, polyethylene glycols, hexanetriol and ethylenediamine.

If the original plan is followed, the next full volume of 'Meyler' is likely to appear soon. However the success of the 'Annuals' project and the ease with which these supplements can be used have made the need for new volumes less urgent.

Quality Control in Toxicology. Edited by G. E. Paget. MTP Press Ltd., Lancaster, 1977. pp. xiii + 128. £9.50.

In 1977 the second in a series of symposia entitled "Topics in Toxicology" was organized by Inveresk Research International. On this occasion the topic, "Quality Control in Toxicology", brought together scientists and regulatory authorities from various parts of Europe and North America to discuss aspects of the quality control of laboratory practice. The papers presented at this meeting have been published in the volume named above.

There are four main sections—"The Regulatory Viewpoint", "The Industrial Viewpoint", "The Independent Expert" and finally "The Research Contractor"—each with an impressive group of contributors. Writing before the full impact of the US FDA regulations for GLP became evident (the initial proposals were published in the Federal Register in November 1976 and finalized in December 1978), the speakers nevertheless pinpoint the basic problems for those concerned with toxicology. While the need for good laboratory standards is not in dispute, the practical problems of implementing regulated guidelines and of subsequent laboratory inspection for compliance are clearly outlined in all of the chapters, together with the need for moderation lest over-regulation impede scientific development.

It is interesting to read the regulatory view from Swedish, French and American as well as UK contributors, but it is perhaps disappointing that all the chapters concerned with the implementation of GLP in the laboratory are, with one exception, from the UK. While this one exception will in itself hold considerable attention for many involved with GLP, being a contribution from Searle Laboratories, USA, on the background to 'the basis and need' for GLP regulations, the remainder become very familiar in content and tend to serve merely as platforms for the advertisement of each company's method of compliance.

In general, the view of the implications of GLP provided by this book seem rather naïve to those of us involved with the present regulations. This is inevitable in the light of the rapid developments that have taken place, but unfortunately it prevents a clearly presented and precisely written book from offering much that is new or unsaid in this area.

Standard Operating Procedures in Toxicology. Edited by G. E. Paget and R. Thompson. MTP Press Ltd., Lancaster, 1979. pp. xviii + 650. £39.00.

Concern for the freedom of scientific thought and development was expressed by toxicologists following the publication of the FDA proposals for Good Laboratory Practice. While agreeing that only the highest possible standards of laboratory practice are acceptable, many do not believe that standards should be dictated by a rigid set of rules. However the FDA has given its assurance that the regulations should be regarded as flexible guidelines only. They should thus allow for the development and progress of good science and are not intended to regulate or interfere with individual company policy.

On the face of it, therefore, it is surprising to find a large publication consisting solely of written Standard Operating Procedures, when the whole concept of each laboratory's procedure is that it is specific to that laboratory. It is indeed an irksome task to commit to paper every detail for every process within a facility, especially for washing and cleaning techniques, but these documents, once embarked upon, may be valuable as staff training manuals and for ensuring that techniques passed on from one generation of technicians to the next are correct and consistent with the idiosyncrasy of that laboratory.

Following a very brief introduction, the first, and smallest, section of this volume is devoted to the Inveresk Research International (IRI) Code of Good Laboratory Practice, comprising documents that set out company policy regarding the general areas of laboratory management such as personnel, protocols, the receipt of animals, data recording, report production and so on. In the introduction it is pointed out that certain company- and instrument-specific SOPs have been ommitted, but for the rest IRI have divided their SOPs generically as far as is practicable, grouping them under the headings Test Substance, Record Keeping, Quality Assurance, 'General' Toxicology, Pathway, Clinical Chemistry and Development Toxicology Procedures. The remaining 589 pages of the book are devoted to SOPs for record keeping, the handling of test substances and 'general' toxicology procedures. The latter section, which is subdivided into procedures that are generally applicable to test animals irrespective of species and those concerned specifically with the mouse, rat, rabbit, dog or primate, is a fully comprehensive set of documents pertaining to every facet of animal husbandry. It is assumed that the three remaining groups of SOPs will make up the companion volume on Pathology, not yet available.

The authors suggest that their publication will save time and effort by providing others with a basis for compiling SOPs, but any scientific establishment committed to the safety evaluation of chemical compounds must organize its own procedures to a suitable format and develop a method for monitoring the results.

The last thing that is wanted is a rigid approach to unnecessary regulations, more particularly when the regulatory authority itself has declared against rigidity. This expensive publication can only propagate an unthinking approach to toxicology.

Analysis of Drugs and Metabolites by Gas Chromatography-Mass Spectrometry. Vol. 1. Respiratory Gases, Volatile Anesthetics, Ethyl Alcohol and Related Toxicological Materials. By B. J. Gudzinowicz and M. J. Gudzinowicz. Marcel Dekker, New York, 1977. pp. vii + 223. Sw. fr. 78.00.

This volume is a comprehensive discussion of a very narrow area. Its two chapters "Respiratory gases, volatile anesthetics, and related toxicological materials" and "Ethyl alcohol and volatile trace components in breath, body fluids, and body tissues" cover the gas-chromatographic analysis of a range of compounds in extreme, and sometimes confusing, detail.

Some pharmacological and pharmacokinetic comments add interest to an otherwise dull text, but the coverage of gas chromatography–mass spectrometry is so sparse that it hardly justifies the serious title.

Excellent features include a wealth of diagrams, both of real gas-chromatography traces and of equipment construction, and a comprehensive index. Some further breakdown of the index would be valuable, however. No less than 47 page numbers are listed after 'flame ionization' for instance, and most of them contain no information of any significance on FID.

This book collects together a great deal of information on the analysis of biological fluids, gases and tissues for a range of compounds, including ethanol, fluorocarbons, solvents and riot-control irritants. Data relevant to the monitoring of gas mixtures, such as those used for the sterilization of medical equipment or the fumigation of stored food materials, are also presented.

BOOKS RECEIVED FOR REVIEW

Developments in Sweeteners—1. Edited by C. A. M. Hough, K. J. Parker and A. J. Vlitos. Applied Science Publishers Ltd., London, 1979. pp. xii + 192. £15.00.

Environmental Carcinogens—Selected Methods of Analysis. Vol. 2. Methods for the Measurement of Vinyl Chloride in Poly(vinyl chloride), Air, Water and Foodstuffs. By D. C. M. Squirell and W. Thain. IARC Scientific Publications No. 22. International Agency for Research on Cancer, Lyon, 1978. pp. xiii + 142. Sw. fr. 75.00 (available in the UK through HMSO).

Carbon Disulfide, Environmental Health Criteria 10. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1979. pp. 100. Sw. fr. 10.00 (available in the UK through HMSO).

Foreign Compound Metabolism in Mammals. Vol. 5. A Review of the Literature Published during 1976 and 1977. Senior Reporter D. E. Hathway. The Chemical Society, London, 1979. pp. xv + 567. £32.00.

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Information Section

ARTICLES OF GENERAL INTEREST

SACCHARIN CONTAMINANTS IN FOCUS

To the controversy over the long-term effects of saccharin, and particularly over the significance to man of the bladder tumours induced by saccharin in rats under appropriate experimental conditions, have recently been added demonstrations that saccharin is possibly mutagenic both *in vitro* and *in vivo* (*Cited in F.C.T.* 1979, **17**, 91). The debate has drawn attention to the variety of impurities in commercial saccharin, and a group responsible for an earlier study on the metabolism of saccharin (*ibid* 1978, **16**, 293) has now produced a series of papers on the disposition and fate of six impurities.

The levels and identity of the contaminants vary widely according to the process used to produce the saccharin. One well-known impurity is toluene-2-sulphonamide (*o*-toluenesulphonamide; OTS), which has been detected at levels as high as 5000 ppm in some samples of saccharin produced from toluene by the Remsen–Fahlberg process and has also been found in much smaller amounts (about 2·5 ppm) in some batches produced by the Maumee process (Stavric, *87th Annual Meeting Ass. off. analyt. Chem.*, Washington, USA, 1973). This compound and its *p*-isomer (toluene-4-sulphonamide; PTS), which is also present in saccharin produced by the Remsen–Fahlberg process, are among the saccharin contaminants included in the recently reported metabolic studies, which were carried out principally in the rat, but to some extent also in man.

The excretion of PTS and of 4-sulphamoylbenzoic acid (4-SBA) was studied in Wistar rats by Ball *et al.* (*Xenobiotica* 1978, **8**, 183). When [*Me*-^{14}C]PTS was given intragastrically to these rats in a dose of 29 or 200 mg/kg, 66–89% of the label appeared in the urine and 2–8% in the faeces within 5 days, most of it in the first 24 hr. The activity in the faeces was in the form of 4-SBA, which therefore appears to be a tissue metabolite of PTS, since the gut flora does not effect this conversion. The major urinary metabolite (93% of the urinary ^{14}C) was also 4-SBA. Other compounds appearing in the urine were unchanged PTS (1·5–2·3%), 4-sulphamoylbenzyl alcohol (2–4%), 4-sulphamoylbenzaldehyde (up to 1·5%) and, after the 200-mg/kg dose, *N*-acetyltoluene-4-sulphonamide (about 2%). Oral administration of (*carboxy*-^{14}C)-labelled 4-SBA in a dose of 22 mg/kg was followed by excretion of 94% of the label within 24 hr, a major part (about 74% of the dose) again being in the urine. Recovery was complete within 6 days, and the appearance of some 25% of the administered dose in the faeces during this period was attributed to incomplete absorption. 4-SBA was excreted unchanged and only 0·01% of the ^{14}C label appeared in the expired air. The indications are, therefore, that prolonged consumption of saccharin containing PTS or 4-SBA will not lead to tissue accumulation, unless the oxidation of PTS and the renal handling of the resulting 4-SBA are impeded for any reason.

Extending this work to the toluenesulphonamide isomer OTS, Renwick *et al.* (*ibid* 1978, **8**, 461) reported that 92% of an oral dose of 20 mg [*Me*-^{14}C]OTS/kg was excreted by rats in 24 hr, 88% being recovered, on average, from the urine and 4·5% from the faeces within 7 days. Higher doses were less rapidly eliminated, the proportion of single doses of 125 and 200 mg/kg exceeded in 24 hr being only 70 and 43% respectively. Excretion of the label in the faeces (4·4 and 4·2%) was not affected by the dose, but urinary excretion accounted for only some 58 and 36% of the dose, on average, at this time, indicating some overloading of the metabolic process or of the renal elimination of the metabolites. By day 7, however, urinary excretion had accounted for 63–85% of both doses, with mean total elimination rates of 87 and 90%. A slower rate of elimination was demonstrated in man. In volunteers given only 0·2 or 0·4 mg [*Me*-^{14}C]OTS/kg. some 56% of the activity appeared in the urine in the first 24 hr and 80% within 48 hr. Faecal excretion was negligible. With the 0·2 mg/kg dose, excretion was virtually complete by day 4, while 88% of the higher dose had been accounted for in the urine by day 7. In the urine of both rats and man, the main metabolites of OTS were 2-sulphamoyl alcohol and its sulphate and glucuronide conjugates (accounting together for about 80% of the urinary ^{14}C in rats and some 40–50% in man) and saccharin (3% in rats and 35% in man). Urinary 2-sulphamoylbenzoic acid (2-SBA) accounted for 2% of the urinary ^{14}C in rats and for about 4% in man, *N*-acetylOTS for 6 and 2%, respectively, and unchanged OTS for 6 and 3%, respectively.

A further impurity in commercial saccharin, benz[*d*]isothiazoline-1,1-dioxide (BIT) was investigated by Renwick & Williams (*ibid* 1978, **8**, 475), who treated female rats with 40 or 400 mg [3-^{14}C]BIT/kg by gastric tube or by ip injection. In all cases, recovery of the label was essentially complete within 1 wk. Rats given the lower dose eliminated 97% of the label within 24 hr, 92% appearing in the urine. Oral and ip doses of 400 mg BIT/kg showed 45–60% elimination within 24 hr, with 42–53% in the urine. Faecal excretion after the ip dose or after an oral dose of 40 mg BIT/kg totalled no more than 2–4%, but incomplete absorption of the larger oral dose was indicated by the appearance of 12% in the faeces. Labelled compounds in the urine were saccharin (about 30% of the urinary ^{14}C), 2-SBA (35%) and 2-sulphamoylbenzyl alcohol (15%), together with the unchanged com-

pound (5–10%) and a polar labile metabolite, which yielded BIT on acid hydrolysis. In man, about 93% of the ^{14}C activity was eliminated in the urine after a dose of 0·5 mg BIT/kg, and less than 1% was recovered from the faeces (Renwick & Williams, *loc. cit.*). Urinary excretion amounted to 59% within 6 hr and 80% within 12 hr. Urinary metabolites of BIT in man included saccharin (about 50% of the urinary ^{14}C), 2-SBA (7%) and 2-sulphamoylbenzyl alcohol (40% conjugated and 8% free). Excretion of unchanged BIT in man was negligible, and only traces of a polar labile metabolite appeared.

3-Aminobenz[*d*]isothiazole-1,1-dioxide (ABIT) and 5-chlorosaccharin (CS) may be present as minor impurities in commercial saccharin, and therefore warranted metabolic studies. Renwick (*ibid* 1978, **8**, 487) reported that [3-^{14}C]ABIT given orally to rats in a dose of 25 mg/kg was rapidly eliminated in the urine (87% in 24 hr and 89% in 4 days), with only 2% appearing in the faeces. Only 0·6% of the ^{14}C administered was detected in the tissues 4 days after treatment. Of the ^{14}C label in the first 24-hr urine, 99% was unchanged ABIT, the remainder being an unidentified metabolite. Oral administration of unlabelled CS to rats in a dose of 80 mg/kg was followed by 76% excretion in the urine within 24 hr and 81% within 4 days. Faecal excretion accounted for 4%. The degree of hydrolysis to 5-chlorosulphamoylbenzoic acid was insignificant (0·3% at most). Thus, provided that renal excretion of these two compounds is not affected by the presence of relatively large amounts of saccharin, neither is likely to accumulate in the tissues of saccharin consumers.

These studies have demonstrated that both PTS and OTS are readily absorbed following ingestion, but whereas PTS is extensively metabolized to 4-SBA and only traces of the intermediate alcohol and aldehyde are excreted, the major excretory products of OTS are 2-sulphamoylbenzyl alcohol and its conjugates, further oxidation of the alcohol to the aldehyde and to 2-SBA being very limited. Acetylation of the sulphonamide group, shown to occur to a limited extent with both PTS and OTS, is a relatively uncommon reaction. A further metabolite of OTS is saccharin, which was identified as a minor metabolite in the rat and a major metabolite in man, demonstrating a species difference in the relative importance of the necessary cyclization process. Subsequent studies with BIT suggested that this cyclization probably resulted mainly from interconversion between 2-sulphamoylbenzaldehyde and 3-hydroxyBIT (Renwick & Williams, *loc. cit.*).

Renwick (*loc. cit.*) concludes from the speed of excretion and identity of most of the saccharin contaminants and their metabolites, from the absence of aromatic ring hydroxylation and *N*-oxidation—reactions implicated in the carcinogenicity of polycyclic aromatic hydrocarbons and aromatic amines, respectively—and from results available from some carcinogenicity studies, that most of the known contaminants are unlikely to be implicated in tumour induction. He suggests that BIT is the only contaminant worthy of further investigation in this connection. This compound produces a labile urinary metabolite in the rat, but not in man, a point that may be of some interest since Radomski & Brill (*Arch. Tox.* 1971, **28**, 159) have shown that labile conjugates play an important role in the carcinogenic action of aromatic amines on the bladder wall.

[P. Cooper—BIBRA]

VINYL CHLORIDE—PART 4: CARCINOGENICITY IN MAN

Although, it is now recognized that vinyl chloride (VC) is carcinogenic in man, the degree of risk involved has still to be established. With a major industrial chemical man acts as his own experimental animal. Consequently, the epidemiological studies of VC now being undertaken will provide the principal basis for an assessment of the carcinogenic risk.

Angiosarcoma of the liver is a tumour seen only rarely in the general population. The association between VC exposure and angiosarcoma in both laboratory animals and man has promoted an otherwise esoteric corner of pathology into the centre of the stage. Since pathology is to some extent a subjective science, the interpretation of data demands great care. A study carried out by the HSE Employment Medical Advisory Service, EMAS (Baxter *et al. Br. med. J.* 1977, **2**, 919) indicates some of the problems involved. In the decade finishing in 1973, only 41 death certificates mentioning angiosarcoma of the liver (or one of the five accepted synonyms) were received by the Registrars General of England, Wales and Scotland (figures for Scotland were not available for the first 2 yr of the decade). These 41 subjects comprised 14 women and 27 men, including one female and two male infants. A search of the medical literature revealed nine cases, five of whom were not included in the above group since they had diagnoses other than angiosarcoma on their death certificates. One additional case was reported directly to EMAS. Adequate histological sections obtained from 33 (70%) of the identified cases, were reviewed by a panel of three or four histopathologists. The diagnosis of angiosarcoma was considered acceptable in only 14 cases, seven cases were considered doubtful and 12, in the opinion of the panel, had been wrongly described. In the 14 instances in which angiosarcoma was confirmed and an occupational history was available, VC exposure was found definitely in one case and possibly in a second. The former case involved a process worker exposed to monomer levels in excess of 200 ppm for over 20 yr in a PVC-manufacturing plant, and the second was a man who had been employed for 11 yr in a factory in which fabric was coated with PVC, but there was no evidence that he had worked in this part of the factory.

Accurate diagnosis of angiosarcoma seems equally uncertain in the Netherlands (Dalderup, *ibid* 1977, **2**, 1149). The Dutch Factory Inspectorate contacted all

the country's pathology laboratories to obtain information and slides on the cases of angiosarcoma of the liver seen during the period 1950–1975. Twenty-seven cases were identified and in none of these had any association with VC exposure been traced. However, when the histological sections were reviewed by three pathologists, only nine of the original diagnoses were definitely confirmed, with one additional "possible" angiosarcoma.

Developments in the past few years in connection with hepatic angiosarcoma have been reassuring, in that the possible epidemic of cancers that might have been anticipated on the basis of VC's carcinogenic activity in laboratory animals has not materialized. A registry of workers in the PVC/VC industry who develop liver angiosarcoma is being kept by NIOSH in the USA. By October 1977, 70 such cases had been reported from a total of 12 countries. In the 64 cases involving those working in polymerization plants, the median age at diagnosis and the length of VC exposure were 49 and 18 yr, respectively, the median latent period (time from first exposure to diagnosis) being 21 yr (Spirtas & Kaminski, *J. occup. Med.* 1978, **20**, 427). A more detailed report of the ten Canadian cases listed, all of whom had been employed in one VC polymerization plant, which had originally also manufactured the monomer, was published by Delorme & Thériault (*ibid* 1978, **20**, 338).

In an earlier review on VC mention was made of a mortality study of 2100 workers in a South Wales PVC production plant (*Cited in F.C.T.* 1976, **14**, 348). This had apparently shown reassuring results, the investigators concluding that there was no suggestion of a VC-associated increase in the frequency of the more common malignant diseases. The conduct of this study, has, however, been the subject of a number of letters in the medical press. Wagoner *et al.* (*Lancet* 1976, **II**, 194) took exception to the calculation that the 336 men exposed for more than 15 yr to VC had accumulated 6084 man-years at risk, and pointed out that by virtue of their presence in this group, these men could not start accumulating man-years at risk relevant to a period of service of 15+ yr until their sixteenth year at the plant. Their years of service prior to this should have been credited to the two shorter exposure groups of less than 10 yr and 10–14 yr. Reanalysis of the data by Wagoner *et al.* (*loc. cit.*) resulted in a readjustment of the standard mortality ratio (SMR; ratio of number of observed to number of expected deaths $\times 100$) for the various causes of death in the longest exposure group, with consequent increases from 121 to 702 for cancer of the digestive tract, from 62 to 366 for lung cancer and from 73 to 428 for deaths from all types of cancer, indicating a definite pattern of excessive site-specific cancer mortality associated with industrial exposure to VC.

In reply, Duck & Carter (*Lancet* 1976, **II**, 195) accepted the general point made by their critics, but in turn demonstrated a gross underestimate of expected deaths in the revised analysis, and on the basis of their original data recalculated the SMRs. As a result, only cancers of the digestive tract showed an excessively high SMR (of 202) and Duck & Carter (*loc. cit.*) did not think that firm conclusions could be drawn from the limited data involved. Wagoner *et al.* (*loc. cit.*) had evidently applied the death rates derived from a young population (that used in the original study) to an older one—"older" by virtue of the fact that in the case of the group working with VC for more than 15 yr the first 15 yr of exposure were excluded. Duck & Carter (*loc. cit.*) emphasized that a true age-standardized death rate could only be calculated if the age structure were known.

Fox (*Lancet* 1976, **II**, 416) added fuel to the fire by identifying a further weakness in the original report. Dose-response relationships established from studies for which exposure and follow-up periods overlap were said to be of limited value because long-continuing exposure was incompatible with death. Fox (*loc. cit.*) noted that his own study had compared the mortality of men exposed to VC for less than 5 yr with those exposed for 5–9 yr and for 10–14 yr, the follow-up period beginning for all three groups 15 yr after entry into the industry. While an excess of deaths due to liver cancer, especially angiosarcoma, was observed, there was no evidence of an excess of lung and brain cancer in association with VC exposure in the UK. A fuller report of this study (Fox & Collier, *Br. J. ind. Med.* 1977, **34**, 1) noted that 99% of the 7717 men who had worked in the UK PVC manufacturing industry during the period 1940–1974 had been traced. The overall SMR of 75·4 showed a significant reduction compared with national death rates. However, four cases of liver cancer were identified in the exposed group compared with a total expected incidence of 1·64 and two of these tumours (both in men who had been exposed to high VC concentrations, one having died only 8 yr after the first exposure) were classified by independent pathologists as angiosarcoma. Fox & Collier (*loc. cit.*) stressed that since the industry had expanded greatly in the last few years, the full impact might not yet be evident.

In contrast to these findings in the UK, a US study (Waxweiler *et al. Ann. N.Y. Acad. Sci.* 1976, **271**, 40) involving workers from four VC-polymerization plants produced evidence that VC exposure was associated with an increased number of cancer deaths in organs other than the liver. A cohort study was restricted to individuals—1924 in all—who had been exposed originally to VC 10 yr or more before and who had worked on the PVC plant for at least 5 yr. The 35 deaths due to cancer in the 136 men of this group who had died was significantly higher than the 23·5 expected (SMR 149); deaths due to non-malignant respiratory disease were also slightly increased (6 compared with the expected 3·4). When the deaths due to cancer were analysed by site, an excess incidence was found in the central nervous system (3 compared with 0·9), the respiratory system (12 compared with 7·7), the lymphatic and haematopoietic systems (4 compared with 2·5) and the hepatic system (7 compared with 2·5). When the latency period was included in the calculation—the excess risk of death in a cohort of persons who had lived 10 yr or more since first exposure being compared with that of a subgroup who had survived 15 yr since their first exposure—the SMR for all malignant neoplasms was increased to 184 in the older group. Corresponding SMR figures for different types of cancer were 498 for cancer of the central nervous system, 194 for the respiratory system, 176 for the lymphatic and haematopoietic systems and 1606 for the hepatic system. Seven

other cases of biliary or liver cancer and seven more cases of brain cancer were found by the investigators in individuals who had worked at the four PVC plants but had not qualified for inclusion in the cohort study. Nine of the ten brain cancers seen in the whole workforce were histologically confirmed as glioblastoma multiforme.

Is it possible, at the present time, to calculate the likely carcinogenic risk of working with VC or its polymer? Some attempt has been made to relate degrees of exposure to VC to the amounts metabolized and to the incidence of angiosarcoma (*Cited in F.C.T.* 1979, **17**, 424) but the problems are extremely complex. On the evidence of the American epidemiological data (not yet confirmed by findings from the UK), VC is liable to induce tumours in the liver, brain, lung and haemo-lymphopoietic system. The fact that the extrahepatic carcinogenicity has not so far been detected in the British workforce could possibly be connected with the different rates of development of the PVC industry in the two countries. The major growth of the PVC industry did not occur in either the USA or the UK until after the last war. If the latent period of some of the VC-induced cancers proves to be of the order of 25 yr or more, then a true measure of VC's carcinogenic potential may not become apparent until well into the 1980s.

Consequently it is still uncertain whether the present mortality figures accurately reflect VC's carcinogenic activity. Whatever the 1980s have in store, however, it should not be forgotten that the individuals who have developed VC-induced cancers were working in the PVC industry at a time when the levels of monomer in the workroom atmosphere were grossly in excess of those that occur today.

[J. Hopkins—BIBRA]

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ABSTRACTS AND COMMENTS

FOOD ADDITIVES AND CONTAMINANTS

Support for carcinogens from undegraded carrageenan

Watanabe, K., Reddy, B. S., Wong, C. Q. & Weisburger, J. H. (1978). Effect of dietary undegraded carrageenan on colon carcinogenesis in F344 rats treated with azoxymethane or methylnitrosourea. *Cancer Res.* **38**, 4427.

Carrageenans are complex polysaccharides derived from red marine algae. Some undegraded forms are used as stabilizers and thickeners in the food industry. On the other hand, carrageenan derived from the seaweed *Eucheuma spinosum* and degraded by acid hydrolysis to a product with a molecular weight of 20,000–40,000 and a sulphate content of about 30% has been shown to induce colorectal tumours in mice (*Cited in F.C.T.* 1979, **17**, 421).

The effect of a diet containing 15% undegraded carrageenan (Viscarin 402) on the induction of carcinoma by azoxymethane (AOM) or methylnitrosourea (MNU) in rats on a high-fat diet has been reported. Weanling rats were given a semipurified diet with or without 15% carrageenan, and from wk 7 were given 8 mg AOM/kg body weight/wk sc for 10 wk or 2 mg MNU/rat intrarectally twice weekly for 3 wk. The rats were killed for examination 40 wk after the first AOM dose and 30 wk after the first dose of MNU. Untreated control animals had no colonic or other tumours, but one of the thirty fed carrageenan without either carcinogen developed a colonic adenoma, an uncommon lesion in untreated rats. There was a higher incidence of colorectal tumours, as well as more tumours per animal, in those fed carrageenan plus a carcinogen than in those given AOM or MNU alone. Colonic tumours in carrageenan-fed rats given AOM were for the most part situated distally within 4 cm of the anus, whereas those in rats fed control diet with AOM ranged from 2 to 10 cm from the anus. The corresponding MNU-induced tumours occurred within 4 and 7 cm of the anus with the carregeenan and control diets, respectively. The one colonic adenoma in a rat treated only with carrageenan was situated 6 cm from the anus. Tumours were polypoid and sessile, and were larger in rats fed the carrageenan diet than in those exposed to the carcinogen alone.

This work was undertaken because of the interest in dietary fibre and faecal bulk as a possible protection against colon cancer, since carrageenan can absorb larger quantities of water than the commonly used fibres and can act as a bulking agent. In this study, however, undegraded carrageenan enhanced the colorectal carcinogenesis induced by either AOM or MNU in rats.

Urinary nitrosamines

Kakizoe, T., Wang, T.-T., Eng, V. W. S., Furrer, R., Dion, P. & Bruce W. R. (1979). Volatile *N*-nitrosamines in the urine of normal donors and of bladder cancer patients. *Cancer Res.* **39**, 829.

Nitrosamines as a class have been shown to lead to a wide variety of tumours in many animal species. Their formation has been demonstrated in various organs and body fluids (Cooper, *Fd Cosmet. Toxicol.* 1978, **16**, 69) and a recent study demonstrated the presence of volatile nitrosamines in human faeces (*Cited in F.C.T.* 1979, **17**, 415). In the paper cited above, the same group has reported on the presence of these compounds in human urine.

The urine samples were taken from a control group of 27 healthy males, 17 of whom were non-smokers, and from a test group consisting of four patients with bladder cancer. Of these four, one was a non-smoker, one had smoked heavily for over 20 yr and one had smoked for over 30 yr. None of the control or test donors were on any form of medication at the time of the study.

The urine samples were extracted with dichloromethane and the concentrated extracts were analysed for volatile *N*-nitrosamines by gas–liquid chromatography and thermal energy analysis and by high-pressure liquid chromatography. Analysis of urine samples spiked with *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodibutylamine (NDBA) or *N*-nitrosomorpholine (NMOR), in a concentration of 1·0 μg/litre in each case, showed yields of 50, 58, 77 and 90% respectively. Urine samples were taken on more than one occasion from some of the control donors.

No nitrosamines were detected in 11 of the 22 control samples taken from smokers or in 20 of the 28 control samples from non-smokers. Of the remaining smokers' samples, six contained NDMA (0·03–0·10 μg/litre), three contained NDEA (0·09–3·10 μg/litre) and five contained NMOR (0·07–0·67 μg/litre). Of the non-smokers' samples, four contained NDMA (0·02–0·10 μg/litre), three NDEA (0·02–0·05 μg/litre) and four NMOR (0·06–0·17 μg/litre). NDBA was not found in any of the control samples. There was considerable variation in the results for samples taken from the same donor on different evenings. The urine samples from two of the cancer patients contained measurable amounts of NDMA (0·32 and 0·70 μg/litre) and NDBA (0·35 and 0·66 μg/litre), but no nitrosamines were detectable in the urines from the other two patients.

These studies established no relationship between the presence of volatile *N*-nitrosamines in the urine and either smoking habits or the development of bladder cancer. The finding of NDBA, which is a known bladder carcinogen in rats (*ibid* 1965, **3**, 498), was too limited and inconsistent to make any clear contribution to the latter aspect.

The presence of volatile nitrosamines in the urine is not in itself evidence of hazard to the urinary tract, but the authors, who consider that this study and

their earlier work point to formation in the gastrointestinal tract as the most likely source of the urinary nitrosamines, suggest that a similar investigation on much larger groups could be used to study, in particular, the relationship of diet to nitrosamine levels.

FOOD PROCESSING

All clear for irradiated potato

Palmer, A. K., Cozens, D. D., Prentice, D. E., Richardson, J. C., Christopher, D. H., Gottschalk, H. M. & Elias, P. S. (1979). Reproduction and longevity of rats fed on an irradiated potato diet. *Toxicology Lett.* **3**, 163.

In its 1977 report the Joint FAO/IAEA/WHO Expert Committee recommended the unconditional acceptance of wheat and ground wheat products irradiated up to 100 krad and of potatoes irradiated up to 15 krad (*Cited in F.C.T.* 1978, **16**, 63). Information from safety evaluation studies on irradiated foods carried out by the International Project in the Field of Food Irradiation (IFIP) made a very significant contribution to the data on which the committee based its decision. The results of one of those studies are summarized in the paper cited above; the detailed results were described in a final report submitted to IFIP (Palmer *et al.* IFIP Final Technical Report IFIP-R25, 1975).

Potatoes were irradiated with between 8·5 and 15 krad gamma radiation and then were peeled, steamed and used to make up part (35% dry weight) of a balanced diet. Three groups of 70 male and 70 female CD strain rats were given either feed containing irradiated or non-irradiated potato or a normal laboratory animal diet for 30 days. The rats were mated and in each treatment group one F_1 male from each of 30 litters and one F_1 female from each of 60 litters were used for further experiments.

These three groups of 90 F_1 mice were administered the test diets for 11 wk and then mated to produce six successive F_2 litters. The young from five of these litters were weighed and examined for external abnormalities at birth and weighed when 4, 12 and 21 days old. Apart from the males retained for the dominant lethal study, they were killed on day 21 and examined for external and internal abnormalities. Pups in the second F_2 litters were killed at birth for a teratological study. Ten males from the third litters in each group were used for dominant lethal assay and were fed the test diets for 15 wk and then mated with successive sets of untreated females for 4 wk. About 14 days after mating these females were killed and the ovaries and uterine contents were examined. For examination of germinal chromosome morphology, seminiferous tissue anaphase preparations from 30 males in the first litters of each group were examined for chromosomal aberrations.

On completion of the reproductive studies, the F_1 animals were maintained on their respective diets and all surviving animals were killed after 104 wk and examined macroscopically. Organ/body weight ratios were calculated for livers, kidneys and adrenals, and tissue samples were taken for histopathological examination.

Mean litter sizes were generally greater in rats fed either of the potato diets than in the controls. The feeding of irradiated potato had no adverse effects on the parent (F_0) rats or on reproduction in the F_1 generation. The teratological study revealed that the incidences of minor malformations were similar in all groups. An additional teratological study was carried out on three groups of 30 female rats given diets containing 35% non-irradiated potato or 35% potato irradiated with 12 or 50 krad on days 6–20 after mating. The rats were killed and examined on day 20. The number of pregnant rats in both groups fed irradiated potato was slightly higher than that in the group given non-irradiated potato, but there were no other intergroup differences.

No effects of the irradiated-potato diet were observed in the dominant lethal assay, in studies of chromosome morphology or in the longevity study on F_1 rats.

Chlorine dioxide for potable water

Moore, G. S., Calabrese, E. J., DiNardi, S. R. & Tuthill, R. W. (1978). Potential health effects of chlorine dioxide as a disinfectant in potable water supplies. *Med. Hypotheses* **4**, 481.

The FDA has requested further information on the proposed use of chlorine dioxide in water for disinfecting freshly slaughtered carcasses (*Fd chem. News* 1979, **21** (6), 29). The paper cited above reviews available data relevant to the possible effects of chlorine dioxide on human health, since it has been suggested as an alternative to chlorine for disinfecting potable water supplies. Chlorination of water containing organic compounds may produce trihalomethanes and other compounds suspected of having some carcinogenic potential.

One of the possible problems is that the addition of chlorine dioxide to potable water may give rise to chlorates and chlorites. Chlorite has been shown to produce methaemoglobin in rats, probably by a mechanism similar to that occurring in nitrite methaemoglobinaemia. Furthermore, it is considered likely that small amounts of nitrite and chlorite could have a synergistic effect on methaemoglobin formation *in vivo*. The population groups most susceptible to nitrite haemoglobinaemia, namely neonates and persons deficient in glucose-6-phosphate dehydrogenase (G6PD), must also be considered most at risk from the possible effects of chlorine-dioxide treatment of water supplies.

There is, however, a lack of data on which to base a

sound estimate of the actual degree of risk involved, and the author suggests that a suitable model for the necessary animal studies would be one of the inbred mouse strains in which G6PD activity in the circulating erythrocytes is only about 33% of the normal and both the properties and levels of the enzyme are similar to those in individuals with the most common type of G6PD deficiency.

AGRICULTURAL CHEMICALS

More reports of DBCP effects

Potashnik, G., Yanai-Inbar, I., Sacks, M. I. & Israeli, R. (1979) Effect of dibromochloropropane on human testicular function. *Israel J. med. Sci.* **15,** 438.

Following the discovery that dibromochloropropane (DBCP) residues occur on crops and sites previously thought to be uncontaminated, the EPA announced its intention to suspend all uses and all registrations of this pesticide (*Federal Register* 1979, **44,** 43335). Studies in the rat have identified the testis as a target organ of DBCP and industrial exposure to the compound has been associated with reduced or zero sperm counts and loss of sperm motility (*Cited in F.C.T.* 1978, **16,** 498).

A recent morphological study of testicular biopsy specimens from exposed workers (*ibid* 1979, **17,** 555) demonstrated a clear correlation between the duration of DBCD exposure and the reduction in spermatogenic activity. The authors cited above attempted to evaluate testicular function in a further 23 men involved in the manufacture of DBCP. Routine physical and clinical observations, including blood counts and tests for kidney and liver function, reflected no effects of exposure. However, sperm density was in the normal range in only five men, all packers whose exposure time totalled 10–30 hr (or in one case 60 hr). Oligospermia was found in six packers or process operators exposed for totals of 34–95 hr, while the 12 operators with exposure times of 100–6726 hr were all azoospermic. Plasma levels of FSH (follicle-stimulating hormone) were raised in the azoospermic group, as in the California study (*ibid* 1978, **16,** 498), but levels of luteinizing hormone as well as testosterone and thyroxine levels were generally unaffected. Testicular biopsies from these men showed no evidence of active spermatogenesis and there was complete atrophy of the seminiferous epithelium, most tubules being lined only with Sertoli cells. Groups of tubules were hyalinized but the Leydig cells in the interstitial tissue appeared normal.

Unfortunately atmospheric levels of DBCP could not be determined, so the severity of exposure for different types of worker was not established. Moreover no conclusions could be drawn regarding possible reversibility of the effect, since although some azoospermic men had not been working directly with DBCP for 1–5 yr, they were still working on the site at the time of the study.

DDT, dieldrin and the quail

Shellenberger, T. E. (1978). A multi-generation toxicity evaluation of P,P′-DDT and dieldrin with Japanese quail. I. Effects on growth and reproduction. *Drug Chem. Toxicol.* **1,** 137.

Shellenberger, T. E. & Fullerton, F. R. (1978). A multi-generation toxicity evaluation of P,P′-DDT and dieldrin with Japanese quail. II. Tissue residues analyses. *Drug Chem. Toxicol.* **1,** 147.

These papers describe the effects of dieldrin and *p,p′*-DDT on Japanese quail in a four-generation (parental, F_1, F_2 and F_3) study carried out on ten groups of 21 Japanese quail chicks, between 3 and 5 days old. *p,p′*-DDT (at 5 and 50 ppm) and technical dieldrin (0·1 and 1·0 ppm), mixed into game-bird feed, were each fed to two groups of birds. The remaining two groups were used as controls and given untreated feed. The birds were dosed for 10 wk and, during this period, growth, feed consumption, deaths and egg production were recorded. Eggs laid during wk 9 and 10 were put into incubators and the number hatching within 20 days was recorded. Any eggs unhatched after this time were broken and examined for fertility. The newly hatched chicks (20–35 chicks/group) were fed the same experimental diets for 10 wk, except for F_3-generation chicks which were fed for only 5 wk. After each 10-wk feeding period, surviving birds were fed on pesticide-free diets for 12 wk, and body weight, feed consumption, egg production and egg viability were measured during the recovery period.

The onset of egg production appeared to be delayed in F_1 birds fed 1·0 ppm dieldrin, but by the end of the pesticide-feeding period egg production was normal. F_2 birds fed 50 ppm DDT produced fewer eggs that hatched (57·9%, compared with 70·0% for the control) and this seemed to reflect lower egg fertility, which was 79% in this group, compared with 95% in the controls. However, the author comments that these effects may have been due to the variations between birds commonly observed in this species. No other effects of the pesticides on the growth, egg production or mortality of birds were noted.

Birds were killed at intervals during the experiment and samples of brain, liver, muscle, gonads and fat were analysed for pesticide residues or metabolites by gas chromatography. Samples of egg yolks were also analysed. DDT residues (including DDD and DDE) were found mainly in the fat. During the 12-wk recovery period the levels of DDT residues in the tissues decreased, the decrease being more rapid in female birds. Total DDT residues in individual tissues were similar in each generation but the residues found in the brain, liver and muscle of newly-hatched chicks were several times greater than those found in the same birds 5 wk later. In egg yolks, dose-related increases in DDT residues occurred during the pesticide-feeding period and the residue levels in eggs laid during the recovery period decreased, returning to control levels in the case of birds given 5 ppm DDT. There were no generation differences in DDT levels in yolks.

Similar effects were observed in dieldrin-dosed birds, although detailed egg-yolk analyses were not carried out. As with DDT, dieldrin residues were carried over to successive generations but the levels declined during the pesticide-feeding period until they were similar to those of previous generations.

Another catastrophe for ETU?

Khera, K. S. & Iverson, F. (1978). Toxicity of ethylenethiourea in pregnant cats. *Teratology* **18,** 311.

In recent years, interest in ethylene thiourea (ETU) has grown as a result of reports of its mutagenic, carcinogenic and teratogenic effects (*Cited in F.C.T.* 1979, **17,** 177). Of these effects, the teratogenic response and its underlying mechanisms appear to have been the most investigated. ETU has already been shown to induce foetal malformations in both rats and rabbits (*ibid* 1974, **12,** 282). The above-cited study examines the effects of the compound on pregnant cats.

ETU was administered orally to time-mated cats in daily doses of 0, 5, 10, 30 or 60 mg/kg days 16–35 of gestation or of 120 mg/kg from day 16 until the appearance of toxic effects (between days 27 and 34). The cats given daily doses of 10, 30, 60 or 120 mg ETU/kg showed signs of toxicity which were delayed in onset and characterized by progressive loss of body weight, ataxia, tremors and hind-limb paralysis. Those that died, or were killed when moribund yielded, at autopsy, 35 live foetuses, 11 of which were malformed. Coloboma and umbilical hernia each occurred in four foetuses, cleft palate in two and spina bifida in one.

The incidence of abortion was unrelated to treatment, and the pregnant cats that remained healthy or outlived toxicosis were killed on day 46. There was no evidence that ETU affected foetal viability or foetal weight. Two anomalous foetuses were observed in these litters: one, from the 5 mg/kg group, had exencephaly, hydrocephaly and cleft palate, and the other, from the 120 mg/kg group, had kyphoscoliosis and umbilical hernia. Other minor skeletal anomalies occurred, such as an excess of rib pairs and a reduction in sternebrae but the significance of these anomalies was unknown.

The results indicate that in the cat, the central nervous system is the site most susceptible to the toxic effects of ETU. Foetuses from cats in a moribund state due to ETU toxicosis showed a high incidence of malformations, but it was uncertain whether these malformations resulted from a primary effect of ETU on foetal development or from indirect effects of the maternal toxicosis. However, no clear evidence for the teratogenicity of ETU was observed in live foetuses from cats surviving up to day 46 of gestation.

[Such is the concern over the effects of ETU, that NIOSH has prepared an occupational hazard review (*NIOSH—Special Occupational Hazard Review with Control Recommendations for Ethylene Thiourea*, by A. R. Gregory; DHEW (NIOSH) Publication No. 79-109, U.S. Department of Health, Education and Welfare, Washington, 1978; pp. vi + 76). The document advises that ETU should be handled in the workplace as if it were a human carcinogen and teratogen and that occupational exposure should be minimized.]

Stomach tumours from nitrosocarbamates

Lijinsky, W. & Schmähl, D. (1978) Carcinogenicity of *N*-nitroso derivatives of *N*-methylcarbamate insecticides in rats. *Ecotoxic. envir. Safety* 2, 413.

A recent study has shown that *N*-nitrosocarbaryl may be formed when the insecticide carbaryl (*N*-methyl-1-naphthyl carbamate) is incubated with sodium nitrite in rat gastric juice *in vitro* (Beraud *et al. Fd Cosmet. Toxicol.* 1979, **17,** 579). The nitrosation of carbaryl with nitrite in aqueous solution at low pH has been reported previously and there is evidence that *N*-nitrosocarbaryl is a potent mutagen in bacteria and induces stomach tumours in rats (*Cited in F.C.T.* 1977, **15,** 500). Nevertheless, a limited study in which rats were given orally both carbaryl and nitrite failed to demonstrate any direct or transplacental induction of tumours (Lijinsky & Taylor, *Fd Cosmet. Toxicol.* 1977, **15,** 229).

Two of the authors concerned independently with the earlier *in vivo* studies have now published jointly their further work on tumour induction in Sprague–Dawley rats treated orally with the *N*-nitroso derivatives of a range of *N*-methylcarbamate insecticides, including carbaryl. Unless high toxicity of the test compound necessitated some modification, young rats in each study were intubated weekly for 10 wk with doses that were the molar equivalents of the weekly nitrosocarbaryl dose (60 mg/kg body weight in corn oil in one test and in the other 0·2 ml of an olive-oil solution containing 25 mg/ml). The higher dose level was given to males and females but only females were used for the study in which the lower doses were given. In both tests the animals were kept until they died.

No stomach tumours were found in males treated with corn oil alone (apparently the only control group included), but some rats from each of the test groups developed tumours of the forestomach, and with some compounds the proportion affected was high. Although poor survival in groups given the more toxic compounds (nitrosocarbofuran, nitrosobaygon, nitrosomethylphenyl carbamate and nitrosoaldicarb) made evaluation difficult, the authors identify the nitroso derivatives of aldicarb, baygon, carbofuran and alandrin as the most potent carcinogens, followed by nitrosocarbaryl and then by the methomyl and Bux-ten derivatives.

The tumours, many of them carcinomas, occurred only in the forestomach. The absence of induced tumours at other sites suggests that there may be little, if any, absorption, since these compounds have been shown to be extremely potent bacterial mutagens without metabolic activation. It is notable, however, that the apparent order of carcinogenic potency demonstrated for these compounds was the reverse of their relative mutagenic potential in bacterial systems, nitrosobaygon and nitrosocarbofuran having been found to be the weakest mutagens and nitrosomethomyl the most potent.

Ethoxyquin distribution in rats

Skaare, J. U. & Nafstad, I. (1979). The distribution of ^{14}C-ethoxyquin in rat. *Acta pharmac. tox.* 1979, **44**, 303.

Ethoxyquin (EQ), used as an antioxidant in animal feeds, particularly those containing fish meal, and for controlling scald in apples and pears, has been found in rats to be nephrotoxic in high doses and in some circumstances to cause liver enlargement, ultrastructural hepatic changes and enzyme induction or suppression (Skaare *et al. Toxic. appl. Pharmac.* 1977, **42**, 19). The liver enlargement and enzyme induction associated with dietary administration of EQ have been classified as an adaptive rather than toxic response on account of their reversibility (*Cited in F.C.T.* 1976, **14**, 67). The observed capacity of EQ to protect rats against the hepatotoxicity or carcinogenicity of several compounds has been attributed to its ability to induce some liver enzymes and suppress others (*ibid* 1979, **17**, 306). Its effects on enzyme systems vary, however, with dose levels and route of administration, and may also be responsible for EQ's capacity to enhance the hepatotoxicity of *N*-nitrosodimethylamine.

Early work (Wilson *et al. J. agric. Fd Chem.* 1959, **7**, 206) showed that after oral administration of EQ to rats, the compound and its metabolites were rapidly and almost completely excreted, but in view of the growing reports of its activity, further information on the distribution and possible accumulation of EQ in rats is clearly of interest.

Whole-body autoradiography was performed on rats killed at intervals between 0·5 and 144 hr after intubation with a single dose of ^{14}C-labelled EQ (104 mg EQ/kg; 250 μCi/kg). At 0·5 hr the radioactivity was present in most of the body except the central nervous system, the level of activity, apart from that in the gut, being greatest in the liver (about 2·2% of the dose) and in the renal pelvic cavity and renal cortex. For 4–8 hr after the dose was administered, there was a general increase in tissue radioactivity, particularly in the adipose tissue and in mucosal cells. The radioactivity in the liver reached a peak by 8 hr and thereafter fell relatively slowly, still accounting for 0·5 and 0·2% of the dose after 2 and 6 days respectively, so that 7·5% of the peak hepatic level was still retained 6 days after dosing. This suggests an important role for the liver in EQ metabolism. Biliary excretion of EQ or its metabolites and/or their possible accumulation in the intestinal wall was indicated by the absence of radioactivity from the stomach after about 20 hr although a high level of ^{14}C persisted in the intestine.

By 48 hr the general level of radioactivity had declined, the main sites of retention apart from the liver being the intestines, renal medulla and blood. By day 6, renal excretion of radioactivity had been drastically reduced, but the level of activity remained high in the renal cortex, an interesting finding in view of the reported nephrotoxicity of EQ. The relatively slow rate of excretion found in this study may have been due to the tendency of the antioxidant to accumulate in adipose tissue, from which it is only slowly released.

OCCUPATIONAL HEALTH

Arsenic metabolism in the dog

Charbonneau, S. M., Tam, G. K. H., Bryce, F., Zawidzka, Z. & Sandi, E. (1979). Metabolism of orally administered inorganic arsenic in the dog. *Toxicology Lett.* **3**, 107.

Epidemiological studies of humans exposed to inorganic arsenic have revealed a correlation with an increased incidence of lung, lymphatic and skin cancer (*Federal Register* 1975, **40**, 3392). Angiosarcoma of the liver has also been reported as a result of exposure to arsenical compounds (*Cited in F.C.T.* 1976, **14**, 507). However, many investigations in rodents have failed to reproduce these observations, a discrepancy that may reflect marked differences in absorption, excretion and tissue binding in the rat and man. It has been suggested that methylation is involved in the detoxication of inorganic arsenic in mammals, at least in cows and dogs (*ibid* 1976, **14**, 507). Recent studies by the group cited above (report in press) have indicated that the pharmacokinetics of arsenic in the dog following iv injection are similar to those reported for man. In the work reviewed here, this group has examined further the metabolism of arsenic in the dog in an attempt to clarify events in man.

A single oral dose of ^{74}As (approximately 0·50 mCi and 0·2–0·6 μg arsenic/dog) was administered as arsenic acid to male and female dogs. At 40-min intervals for up to 6·5 hr, blood and urine samples were taken and analysed for arsenic metabolites. The dogs were maintained on a diet that provided a daily intake of unlabelled arsenic of about 40 μg/day.

The major metabolite, dimethylarsinic acid (DMAA), was detected in the plasma and red blood cells within 50 min of dosing. The maximum level of arsenic was reached in the blood about 2 hr after dosing. Initially the total amount of arsenic in the plasma was greater than that in the red blood cells, but after 4·5 hr this situation was reversed. Initially most of the arsenic present in the urine was in inorganic form, but within 2 hr more than 80% was DMAA. By 6 hr, DMAA accounted for more than 90% of the arsenic in the plasma, red blood cells and urine. No significant amounts of methylarsonic acid were detected in either the blood or urine, although it has been detected, with larger quantities of DMAA, in human urine after arsenic exposure. Its absence probably reflects the rapidity of the methylation reaction in the dog. The investigators suggest that the erythrocytes as well as the liver may be involved in this methylation. These studies also indicated that arsenic does not bind to red blood cells in the dog, as has been observed in the rat, and confirmed the similarity between the erythrocyte/plasma ratio of arsenic in the dog and man.

Monitoring chromium nephrotoxicity

Franchini, I., Mutti, A., Cavatorta, A., Corradi, A., Cosi, A., Olivetti, G. & Borghetti, A. (1978). Nephrotoxicity of chromium. Remarks on an experimental and epidemiological investigation. *Contr. Nephrol.* **10**, 98.

Previous experimental studies with rats have shown that in chromate nephrotoxicity the amount of urinary lysozyme increases as damage to the proximal tubule becomes more marked (*Cited in F.C.T.* 1976, **14**, 71). Using this and other urinary indicators of renal-tubule damage, an attempt was made in the above-cited study to define more closely the mechanisms responsible for the toxic effects of chromium in rats and to relate them to observations made on a group of chromium-exposed workers.

In an acute study of chromium toxicity, female Wistar rats received potassium dichromate in a single sc injection (15 mg/kg body weight) and the effects were determined during three periods after dosing (1–8, 8–24 and 24–48 hr). The glomerular filtration rate remained constant and within normal limits throughout the observation period. The chromium concentrations in the serum ultrafiltrate and urine were considerably higher than those found in control subjects and were progressively reduced. Chromium clearance and the excretion fraction were significantly higher than in the controls and remained constant throughout the 48 hr. In addition, the median values for urinary indicators of cellular lesions (β-glucuronidase) and of altered tubular metabolism (proteinuria, lysozymuria and glucosuria) were significantly higher than in the controls and increased progressively. The functional alterations observed corresponded to various renal morphological changes; there was a gradual appearance of cytoplasmic vacuolization, and complete cellular necrosis was observed 24 hr after dosing.

In a more prolonged study, 24 female Wistar rats were given 3 mg potassium dichromate/kg body weight every other day for 8 wk, and the renal-function indices were determined weekly. There was a progressive increase in the amount of chromium in the serum ultrafiltrate and an increase in the quantity of chromium excreted in the urine over the treatment period. This was accompanied by a significant increase in chromium clearance and in the fraction of the chromium filtrate excreted. There was also a close correlation between the clearance of chromium and the concentration of the metal in the cortical region of the kidney. No clear relationship was established between increasing degrees of intoxication and the severity of tubular damage, as indicated by proteinuria, urinary β-glucuronidase and lysozymuria, although these were higher on average than in the control animals and the β-glucuronidase levels were intermittently higher even in the early stages of the study.

The levels of β-glucuronidase activity, proteinuria and lysozymuria determined in three groups of chromium-exposed workers were to some extent comparable with the experimental findings. The first group had had only limited exposure (while welding on stainless steel) and showed no evidence of any change in renal function. In the other two groups, who had had greater chromium exposure (in electrode welding on armoured steel and chromium plating), there were strong indications of tubular damage and, in particular, the β-glucuronidase evidence was positive. The electrophoretic pattern of the urinary proteins was also indicative of tubular damage. These effects seem likely to reflect a direct action on the epithelium of the proximal tubule and to be reversible following cessation of exposure and repair of the epithelial lesion.

Microsomal chromate metabolism

Gruber, J. E. & Jennette, K. W. (1978). Metabolism of the carcinogen chromate by rat liver microsomes. *Biochem. biophys. Res. Commun.* **82**, 700.

Epidemiological studies have shown an association between exposure to chromium compounds and lung cancer (*Cited in F.C.T.* 1979, **17**, 97). Although most of these studies have implicated hexavalent chromium (Cr^{VI}) compounds there is no conclusive evidence for a particular carcinogenic chromium compound (Sunderman, *Prevent. Med.* 1976, **5**, 279). The metabolism *in vitro* of Cr^{VI} by rat-liver microsomes has now been studied and it is proposed that the ultimate carcinogen of chromium may be the trivalent (Cr^{III}) form.

Microsomal fractions were prepared from the livers of male Sprague–Dawley rats that had been given an ip injection of 20 mg sodium dichromate/kg 16–19 hr before they were killed. The microsomes were incubated with 4×10^{-4} M-chromate (from potassium dichromate) in 0·05 M-TRIS.HCl buffer at pH 7·4 and different concentrations of NADPH were added. Samples were taken at various times, the reaction was stopped by protein extraction and the amounts of Cr^{VI}, Cr^{III} and NADPH were measured spectrophotometrically.

The incubation of chromate with rat-liver microsomes resulted in the reduction of Cr^{VI} to Cr^{III}. The amount of chromate reduced depended on the concentration of the microsomal preparation and the concentration of NADPH. The maximum conversion occurred when $1{\cdot}44 \times 10^{-3}$ M-NADPH was incubated with 1·5 mg microsomes/ml for 6 hr. No reduction occurred in the absence of NADPH, and only a very slow rate of reduction occurred in the absence of microsomes. The results indicate, therefore, that an NADPH-requiring enzyme or enzyme system is involved in the metabolism of chromate *in vitro*.

The authors propose that Cr^{III} is ultimately responsible for the carcinogenic effects of chromium. They base this theory on earlier epidemiological and animal studies and on mutagenicity assays. *In vitro* studies have demonstrated that Cr^{VI} can easily cross cell membranes but Cr^{III} cannot. In cellular assay systems, Cr^{III} has been shown to be mutagenic only at concentrations very much (about 10^3 times) greater than Cr^{VI}. However in a sub-cellular system, Cr^{III} proved to be mutagenic at concentrations much lower than Cr^{VI}. The hypothesis put forward is that Cr^{VI} crosses the cell membrane and may then be reduced to Cr^{III} by a microsomal-enzyme system. The Cr^{III} is capable of binding to proteins and nucleic acids and so may have mutagenic and carcinogenic effects.

[There has been another report of the metabolism

of Cr^{VI} by an NADPH-dependent microsomal enzyme system (Löfroth, *Naturwissenschaften* 1978, **65**, 207). In a Salmonella/microsome test there was reported to be decreased mutagenic activity when Cr^{VI} was reduced to Cr^{III} by the microsomal-enzyme system. However, since the conversion of Cr^{VI} to Cr^{III} was being carried out by microsomal enzymes outside the Salmonella cells, the apparent decrease in mutagenic activity may have been due to the poor uptake of Cr^{III} into the bacterial cells. Obviously further studies are necessary to determine the part played by the microsomal-enzyme system in the carcinogenicity of chromate. Löfroth (*loc. cit.*) also demonstrated that at least one additional factor, as well as NADPH, was required for the reducing reaction in the microsomes.]

Sensitivity of the growing brain to manganese

Dikshith, T. S. S. & Chandra, S. V. (1978). Cytological studies in albino rats after oral administration of manganese chloride. *Bull. env. contam. & Toxicol. (U.S.)* **19**, 741.

Chandra, S. V. & Shukla, G. S. (1978). Manganese encephalopathy in growing rats. *Envir. Res.* **15**, 28.

Manganese (Mn) is used in steel, dry battery and ceramics manufacture. Its addition to petroleum spirit as an antiknock agent poses the problem of environmental pollution. Neurological disorders in workers exposed to Mn are well recognized (*Cited in F.C.T.* 1970, **8**, 468), and neurological degeneration in the rabbit brain has been demonstrated after intratracheal inoculation of Mn dust suspended in saline or after direct injection of $MnCl_2$ solution into the ventricles (*ibid* 1973, **11**, 697). The effects of Mn on chromosomes and on the brain tissues of growing rats have now been investigated.

The first paper cited describes a study in which daily doses of 50 μg $MnCl_2$/kg were given orally to male albino rats for 180 days. The animals were then given 4 mg colchicine/kg ip and killed 4 hr later. Chromosome analysis of femoral bone-marrow and seminiferous-tubule cells was carried out. No significant chromosomal damage occurred in either type of cell; in the bone marrow cells there were a few chromatid gaps and chromatid breaks, but no changes at all were observed in the spermatogonial cells. The mitotic indexes of the bone-marrow and spermatogonial cells from rats treated with $MnCl_2$ did not differ from those of cells from controls.

In the second experiment, the morphological and enzymatic effects of Mn on the growing rat brain were investigated. Male albino rats, 21 days old, were given orally 50 μg $MnCl_2$ in 1 ml water daily for 60 days. Six out of 30 animals died during the experiment, but no cause of death could be established. Survivors showed no behavioural or locomotor disturbances, and no alterations in growth or in brain weights. No gross abnormalities of the meninges, or of the ventricular and vascular systems of the brain occurred. Histological examination revealed focal neuronal degeneration of the frontal cerebral cortex and cerebellar cortex in the brains of Mn-treated rats. These were first apparent after 45 days of dosing and became marked, with neuroglial proliferation, after 60 days. At this time similar changes were observed in the caudate nucleus. Brain monoamine-oxidase activity increased after 15 days of Mn dosage and reached a plateau of maximum activity after 30 days. However, acetylcholinesterase activity was not affected.

It is reported that these morphological and enzymic effects of Mn on the brain tissues of growing rats occurred earlier, and at lower dose levels, than in adult rats in previous experiments. Therefore, although the first study indicates that Mn does not have mutagenic effects, the susceptibility of growing animals to Mn, demonstrated in the second investigation, may be of considerable importance in assessing the environmental hazards associated with this metal.

Nickel cytotoxicity

Adalis, D., Gardner, D. E. & Miller, F. J. (1978). Cytotoxic effects of nickel on ciliated epithelium. *Am. Rev. resp. Dis.* **118**, 347.

Camner, P., Johansson, A. & Lundborg, M. (1978). Alveolar macrophages in rabbits exposed to nickel dust. Ultrastructural changes and effect on phagocytosis. *Envir. Res.* **16**, 226.

Many studies have established a high incidence of respiratory cancer in nickel (Ni) workers (*Cited in F.C.T.* 1966, **4**, 211), and in rats pulmonary carcinomas have been induced by inhalation of Ni carbonyls (Sunderman, *Fd Cosmet. Toxicol.* 1971, **9**, 105). Changes have been found in the nasal epithelium in Ni-exposed workers (*Cited in F.C.T.* 1977, **15**, 362) and increases in the numbers of alveolar macrophages washed from the lungs of guinea-pigs exposed to nickel oxide dust have been reported (Bingham *et al. Archs envir. Hlth* 1972, **25**, 406).

In the first study cited above, cultures of isolated hamster tracheal rings were used to investigate ciliary activity following *in vitro* exposure to different concentrations of Ni chloride and *in vivo* exposures to a Ni aerosol. Excised tracheal rings prepared from male Syrian golden hamsters were treated with basal medium containing 0·65, 6·5 or 65 μg Ni/ml. Fresh medium containing no Ni was used as a control and the ciliary beating frequency was determined after 24, 48 and 72 hr. After 72 hr the rings were fixed and stained for microscopic examination.

The ciliary beating frequency decreased with increasing Ni concentration, regardless of time, and the rings treated with 65 μg Ni/ml showed complete ciliostasis after 24 hr. Dose-related epithelial damage occurred in all rings treated with Ni *in vitro*. Cilial disorganization occurred and the authors suggest that this may be associated with a lack of synchrony of beat and further depression of tracheal clearance.

In *in vivo* experiments, hamsters were exposed to nickel chloride aerosol concentrations of 100 or 275 μg Ni/m^3 air for 2 hr on 1 or 2 days. Tracheal rings were removed from the hamsters immediately after the single or second exposure and put into the medium. The ciliary beating frequencies of rings from control animals were higher than those of rings from Ni-treated hamsters and the effect was dose-related. For a given dose, the effect on beating frequency was significantly greater immediately after the second than after the first exposure.

It is concluded that exposure to Ni lowers ciliary activity both *in vivo* and *in vitro* and leads to epithelial damage. It could therefore impair the respiratory defence mechanisms and thus decrease the resistance of the animal to respiratory infections.

In the second study cited, two groups of four New Zealand rabbits were exposed to metallic Ni dust over a period of 4 wk (5 days/wk, 6 hr/day) at concentrations of 0·45 and 2·04 mg Ni/m^3. A third group was used as a control. Only 40–50% of the Ni dust was respirable (i.e. <7 μm aerodynamic diameter). A few days after the last exposure the lungs were removed from the rabbits. Macrophages were washed out of the right lungs and their phagocytic activity on silver-coated teflon particles and their morphology were determined by light and transmission electron microscopy (EM). Macrophages from a control rabbit were exposed to Ni powder for periods of 0·5–3·5 hr and then fixed for EM.

Lungs from rabbits exposed to Ni were of significantly higher weight and density than lungs from control rabbits. This effect seemed to be dose-related. Increased numbers of teflon particles were phagocytized by macrophages from Ni-exposed lungs and the variances in the diameters of cells washed from the lungs increased with increasing Ni dose. Macrophages from lungs exposed to Ni *in vivo* showed ultrastructural changes, including highly undulating membranes, numerous slender microvilli and protrusions from the cell surface. Macrophages exposed to Ni *in vitro* did not develop slender protrusions X-ray micro-analysis showed that macrophages exposed to Ni *in vivo* did not contain Ni, but those exposed to the metal *in vitro* did.

These differences in the effects of Ni on macrophages *in vivo* and *in vitro* suggest that the observed *in vivo* effects are only indirectly caused by Ni.

Washings from the lungs of Ni-exposed rabbits contained larger amounts of a phosphorus-containing amorphous substance, and extraction with chloroform–methanol (2:1, v/v) indicated the presence of phospholipid. Laminated structures were present in both the macrophages and the amorphous substance washed from Ni-exposed lungs. These laminated structures were similar to the lamellar bodies in alveolar type II cells. Ni-exposed cells containing these laminated structures had fewer primary lysosomes than the control cells.

The authors suggest that the laminated structures in the Ni-exposed macrophages may consist of the amorphous phospholipid-containing substance incorporated into phagosomes and secondary lysosomes. Ni may cause phospholipid secretion from alveolar type II cells and the secreted phospholipid may then affect the macrophages. These effects of exposure to Ni dust are considered to resemble those reported to occur in man and rats exposed to silica dust.

Effects of excessive N_2O inhalation

Layzer, R. B. (1978). Myeloneuropathy after prolonged exposure to nitrous oxide. *Lancet* **II**, 1227.

For many years nitrous oxide (N_2O) was regarded as biologically inert, but recently it has been reported to have adverse effects on animals and man. Among these are megaloblastic bone-marrow changes (Amess *et al. Lancet* 1978, **II**, 339) and damage to spermatogenic cells (Kripke *et al. Anesthesiology* 1976, **44**, 104). There is also concern about the possible teratogenic and embryotoxic effects of N_2O and other volatile anaesthetics (Bussard, *J. Am. dent. Ass.* 1976, **93**, 606). Now neurological abnormalities resembling those of multiple sclerosis have been described in 15 patients, 14 of them dental surgeons, following excessive and prolonged exposure to N_2O. Two were exposed professionally in poorly ventilated surgeries, but 13 had deliberately abused N_2O for periods ranging from 3 months to several years. The neurological symptoms included numbness or tingling in the hands or legs, impaired equilibrium or gait leading to the inability to walk unassisted, impaired sphincter control, impotence and depression. Lhermitte's sign (radiating pain on sudden flexion of the neck) occurred in 12 patients.

Those who had deliberately inhaled N_2O had used concentrations of 30–80%, usually for periods of 30–60 min on 2–7 days every week. Examination during the early stages often showed only reduced tendon reflexes in the legs and impaired sensation of vibration or touch in the feet or hands. Signs of distal sensorimotor polyneuropathy usually appeared later. One patient improved within a few days of discontinuing exposure to N_2O, but in the rest improvement took several weeks or months and recovery was slow. Minor neurological abnormalities persisted in four patients after periods of 6 months to 3 yr, and moderate disability was present in five others 6 wk to 3 yr after cessation of exposure. Six patients relapsed after further exposure to N_2O. The extent of recovery of six patients who were treated with corticosteroids and of four who were given injections of vitamin B_{12} appeared not to have been influenced by the therapy.

Although there is no direct proof that N_2O was responsible for the neuropathy seen in these patients, the indirect evidence points to it, since no other toxic or metabolic cause could be identified and cessation of exposure led, in all cases, to an improvement in condition. It has been suggested that N_2O-interference with vitamin B_{12} metabolism may be implicated in the megaloblastic bone-marrow changes produced by prolonged exposure to the gas (Amess *et al. loc. cit.*). It is possible that some alteration in vitamin B_{12} metabolism by N_2O may also underlie the neurological effects in the cases reported in this study.

Acrylonitrile—focus on hepatic enzymes

Duverger-van Bogaert, M., Lambotte-Vandepaer, M., Noël, G., Roberfroid, M. & Mercier, M. (1978). Biochemical effects of acrylonitrile on rat liver, as influenced by various pretreatments of the animals. *Biochem. biophys. Res. Commun.* **83**, 1117.

Noël, G., Lambotte-Vandepaer, M., Duverger-van Bogaert, M., Roberfroid, M. & Mercier, M. (1978). Hepatotoxic effects of acrylonitrile in rats. *Archs int. Physiol. Biochim.* **86**, 951.

Much attention has been focused on acrylonitrile (AN) following reports that it is carcinogenic in rats and possibly also in man, and on the basis of this evidence the permissible industrial AN-exposure

levels have been considerably reduced in the USA and the UK (*Cited in F.C.T.* 1979, **17**, 554). AN also induces adrenocortical insufficiency, central nervous system toxicity and congestive lung oedema in rats (*ibid* 1979, **17**, 179).

AN is metabolized in animals to cyanide, which is converted to thiocyanate and excreted in the urine. It is thought, however, that this metabolic route is not of sufficient importance to account for the toxic effects of AN (*ibid* 1976, **14**, 74), and that either AN itself, or some other metabolite, plays a part. In the studies cited above the authors investigated the effects of AN on liver enzymes in an attempt to gain further insight into its biological action.

Adult male Wistar rats were given a single ip dose of 3, 10 or 30 mg AN/kg. They were killed at various times after treatment and the blood and livers were removed for analysis. Liver homogenates were fractionated and enzyme assays were carried out on the microsomal fraction.

AN had no significant effect on the activity of glucose-6-phosphatase NADPH-cytochrome *c* reductase, sulphite-cytochrome *c* reductase or of *N*-acetyl-β-glucosaminidase. Neither did it induce changes in the levels of serum glutamic–oxalacetic transaminase or serum glutamic–pyruvic transaminase, and there was no significant lipid peroxidation. However, there were dose-dependent increases in the levels of lactate dehydrogenase (LDH) and sorbitol dehydrogenase (SDH). These increases could be suppressed by pretreatment with either inducers of the cytochrome *P*-450-dependent mixed-function oxidase system (phenobarbital, 3-methylcholanthrene or Aroclor 1254) or L-cysteine. On the other hand, the increases in LDH and SDH were enhanced by the addition of diethyl maleate (DEM), which reduces glutathione levels. AN treatment decreased cytochrome *P*-450 levels and lowered the activities of aldrin oxide synthetase and benzopyrene hydroxylase, but these decreases were not dose-dependent. They were prevented by pretreatment with inducers of the mixed-function oxidase system but were unaffected by DEM. The times of maximum increase in LDH and SDH levels and of maximum lowering of cytochrome *P*-450 levels and aldrin oxide synthetase activities coincided and were about 2 hr after AN treatment.

The changes in SDH and LDH levels induced by 10 mg AN/kg were similar to those induced by 10 mg propionitrile/kg. However, the latter did not affect cytochrome *P*-450 levels or aldrin oxide synthetase activity. This indicates the importance of the vinyl double bond of AN in the mechanism of its reactivity towards cytochrome *P*-450. AN-induced adrenal lesions and deaths can be prevented by prior administration of phenobarbital (Szabo & Selye, *Endocrinol. exp.* 1972, **6**, 141), but phenobarbital pretreatment does not affect the AN-thiocyanate balance (*Cited in F.C.T.* 1975, **14**, 74). It is suggested that the results of this study may be explained by the action of AN, or a metabolite other than cyanide, on cytochrome *P*-450, resulting on the inhibition of the mixed-function oxidase system. Much work remains to be done, however, before the mechanisms of AN metabolism are fully understood.

[The microsomal mixed-function oxidases are thought to play an important part in the metabolism of vinyl chloride monomer (Hopkins, *Fd Cosmet. Toxicol.* 1979, **17**, 404). It is interesting to note, though, that in recent studies carried out in Japanese acrylic-fibre factories there was no indication of disturbed liver function in AN-exposed workers, even in those found to have palpable livers (*Cited in F.C.T.* 1979, **17**, 554).]

Nitrosamines in cutting fluids

Williams, D. T., Benoit, F. & Muzika, K. (1978). The determination of N-nitrosodiethanolamine in cutting fluids. *Bull. env. contam. & Toxicol. (U.S.)* **20**, 206.

Cutting fluids are used in metal cutting and grinding to reduce the temperature of the metal–tool interface; many are synthetic and contain a high proportion of both triethanolamine and sodium nitrite. Fan *et al.* (*Science, N.Y.* 1977, **196**, 70) have reported the presence of levels of 0·02–3% *N*-nitrosodiethanolamine (NDELA) in cutting fluids obtained in the USA. The paper cited above describes similar high levels of NDELA in cutting fluids used in Canada.

Twenty-four samples of commercial cutting fluids were initially screened for NDELA by thin-layer chromatography (TLC). Eight of the nine that contained nitrite also contained NDELA, but in one case it was only a trace amount. The samples containing NDELA were further analysed by gas chromatography–mass spectrometry (GS–MS) and the results were in good agreement with the TLC estimates. Six of the samples contained 0·023 to 0·099% NDELA as determined by GC-MS and one sample contained 0·553%. No NDELA was found by this method in the sample that had shown a trace by TLC analysis. These results are similar to those of Fan *et al.* (*loc. cit.*) and indicate the extent of the problem.

NDELA induces liver tumours in rats (Druckrey *et al. Z. Krebsforsch.* 1967, **69**, 103), but its effects in man are not known. It seems that machinists may be exposed to NDELA through skin-absorption and inhalation. However, synthetic cutting fluids have only been in widespread use for around 20 yr (Fan *et al. loc. cit.*) and because of the latent period involved it is unlikely that any cancer incidence among machinists would at present be related to NDELA exposure. Even so, epidemiological studies to screen workers subjected to NDELA for long periods are clearly needed. The authors point out that industry is apparently seeking to reformulate the fluids where possible to avoid further NDELA exposure.

ENVIRONMENTAL CONTAMINANTS

To take your mind off the price!

Lykke, A. W. J., Stewart, B. W., O'Connell, P. J. & Le Mesurier, S. M. (1979). Pulmonary responses to atmospheric pollutants. I: An ultrastructural study of fibrosing alveolitis evoked by petrol vapour. *Pathology* **11**, 71.

Atmospheric pollutants are associated with two major pulmonary diseases, cancer and bronchitis, which in turn are often associated with emphysema. Since emissions from petrol-burning combustion engines contribute significantly to atmospheric pollution in urban areas, experiments were performed to establish whether their precursor—petrol vapour—also causes pulmonary damage.

Female rats, aged 6–8 wk, were exposed to air containing 100 ppm petrol vapour for 8 hr/day, 5 days/wk for up to 12 wk. The vapour was derived from a commercial 98% octane petrol containing 0·45 g tetraethyllead/litre. Animals were killed at intervals between wk 6 and wk 12. From wk 7 onwards, about half of the exposed rats showed listlessness, rapid breathing and prostration, but those sufficiently distressed to warrant removal from the chamber recovered rapidly and exposure was then resumed. There was little correlation between these signs of respiratory distress and pulmonary changes visible by light microscopy. In fact, such morphological changes were detected unequivocally in only two rats. They consisted of a thickening of the internal structure of the alveolar wall (interstitial thickening), which was focal and irregular in one case and diffuse in the other. Nevertheless, a majority of the other rats exposed to petrol vapour for 6 wk or more showed distinct changes at the ultrastructural level.

Three main types of change were demonstrated by electron microscopy. First to appear, occasionally at wk 6 but more frequently at subsequent kills, were predominantly degenerative changes affecting the fibroblasts and capillary endothelium of the alveolar wall and consisting of vacuolation of the cytoplasm and distension of the endoplasmic reticulum. In the exposure period between wk 6 and wk 10, hypertrophic or hyperplastic changes affecting the Type 2 pneumocytes predominated. These cells became larger, with abnormally numerous dense lamellar bodies, and two nuclei were present in many of the cells in place of the single one normally present. Finally, rats killed between wk 9 and wk 12 generally showed more advanced changes consisting of irregular foci of fibrosis associated with alveolar distortion and collapse. Neighbouring alveolar sacs were distended. The Type 2 pneumocytes in the affected areas lost their lamellar bodies and developed cytoplasmic vacuoles.

[Unfortunately the control animals in this experiment were apparently maintained in a normal laboratory environment and were not exposed to petrol-free air that had passed through the compressor, so some doubt must remain about the exact cause of the changes seen in the treated rats. After compression and expansion, air loses moisture and the effects seen could have been due, at least in part, to the relative dryness of the respired air. Furthermore, petrol may contain a variety of additives, some of which are specific to the brand, and one of these, rather than the hydrocarbons, could have been responsible for the damage. From the pathologist's standpoint, the principal interest of this work lies in the type of cell that was predominantly affected. Usually, pulmonary toxins exert their main effect on the alveolar epithelium, but in this experiment the fibroblast and capillary endothelial cells appear to have been the prime targets.]

Ducks on oil-troubled waters

Szaro, R. C., Dieter, M. P., Heinz, G. H. & Ferrell, J. F. (1978). Effects of chronic ingestion of South Louisiana crude oil on mallard ducklings. *Envir. Res.* **17**, 426.

It has been reported that sea-birds 'cleaned-up' after encounters with crude oil spillages subsequently have a poor capacity for survival apparently because ingested oil affects their growth rate and osmoregulatory mechanism (*Cited in F.C.T.* 1978, **16**, 501). This was demonstrated in gull chicks given a single oral dose of oil, but recent studies on mallard ducklings have demonstrated the results of continued ingestion of small quantities.

There were no treatment-related deaths in groups of mallards fed *ad lib.* a commercial feed containing South Louisiana crude oil at levels of 0·025, 0·25, 2·5 or 5·0% for 8 wk from hatching. Growth was depressed by the diet containing 5% oil and some weight reduction was associated with the 2·5% level. Both of these diets impaired the development of flight feathers. Ducklings on the three higher levels of oil showed impaired avoidance behaviour at day 6, but this was apparently not a general effect on activity levels, since the open-field behaviour of 7-day-olds was not affected.

Tests on blood samples taken after 8 wk showed rises in plasma levels of alanine aminotransferase, originating from the liver, in birds on the three highest dietary levels and of ornithine carbamyl transferase, liberated from the kidney, in all the test groups. Absolute and relative liver weights were increased in birds on the 2·5 and 5% diets, but hepatocyte enlargement was slight and bile-duct proliferation was minimal and limited mainly to the 5% group. Degeneration of kidney tubules also occurred in the latter group. Spleen weights were reduced by the 2·5 and 5% oil diets, which also caused reductions in packed cell volume in the 8-wk blood samples, but no evidence of any osmoregulatory disturbance was obtained from these samples, a possible indication of adaptation to the regular ingestion of oil for 8 wk.

While dietary levels of 2·5–5% for 8 wk are not a realistic reflection of likely environmental conditions, the authors consider that the two lower levels fed (equivalent to about 15 and 150 mg oil/day) could be ingested either directly or via the food chain by ducklings maturing in areas of chronic low-level oil pollution.

More odd alkyl sulphate metabolism

Burke, B., Olavesen, A. H., Curtis, C. G. & Powell, G. M. (1978). The distribution and excretion of non-biodegradable anionic surfactants. *Xenobiotica* **8**, 145.

The biodegradation of alkyl sulphates has been described previously (*Cited in F.C.T.* 1978, **16**, 85). The present study adds to the information regarding the distribution and excretion of such surfactants.

The bile ducts and ureters of anaesthetized adult hooded rats were cannulated. Potassium 10-undecenyl [^{35}S]sulphate ([^{35}S]UDS) in a dose of 5 mg/kg body weight was injected iv into the anaesthetized animals, and bile and urine samples were collected hourly for 6 hr and assayed for total and inorganic ^{35}S. The major route of excretion was the urine. This contained, as mean percentages of the ^{35}S dose, 10·4 and 9·4% inorganic sulphate, and 78·9 and 78·1% total sulphate in males and females, respectively, after 6 hr. In contrast, when the experiment was repeated using potassium 10-phenyldecyl [^{35}S]sulphate ([^{35}S]PDS) in the same dose, most of the radioactivity was excreted in the bile. After 6 hr the mean amounts of the ^{35}S dose excreted in the bile were 8·3 and 10·4% as inorganic sulphate and 43·8 and 73·9% as total sulphate in males and females, respectively. In both sexes an average of 8·9% of the dose was excreted in the urine as inorganic sulphate, whilst urinary total sulphate was 13·7% of the ^{35}S dose in males and 18·8% in females.

Thin-layer chromatography of bile from rats given [^{35}S]UDS showed five radioactive components, one of which was identical to the parent ester. Urine from the same rats showed a major metabolite distinct from the parent ester, as well as inorganic sulphate and trace metabolites representing less than 10% of urinary ^{35}S activity. The major metabolite did not give reactions for a double bond or glucuronic acid, and its identity was not established. Thin-layer chromatography of bile from rats given [^{35}S]PDS showed four radioactive components; one was inorganic sulphate, another was the parent ester, and the other two were identified as glucuronic acid conjugates of the parent ester.

While-body autoradiography and isolated liver perfusion experiments indicated that both of the esters examined became concentrated in the rat liver within 15–30 min of dosing. The study demonstrated that although neither surfactant was degraded to short-chain anionic derivatives, both were considerably metabolized. The chain shortening of alkyl sulphates by ω,β-oxidation can be blocked by certain substitutions of the ω-carbon, the major route of excretion being dependent on the nature of that substitution.

NTA and the mouse

Chu, I., Becking, G. C., Villeneuve, D. C. & Viau, A. (1978). Metabolism of nitrilotriacetic acid (NTA) in the mouse. *Bull. env. contam. & Toxicol. (U.S.)* **19**, 417.

Nitrilotriacetic acid (NTA) is used as a partial replacement for the phosphates in household detergents. Studies in the rat, rabbit, dog and monkey have shown that NTA is rapidly absorbed and eliminated by animals, with some deposition in bone (*Cited in F.C.T.* 1971, **9**, 888). The pattern of elimination was also rapid in man, but there was less absorption from the gastro-intestinal tract than in other species (*ibid* 1974, **12**, 422). The absorption, distribution and excretion of NTA in the mouse were determined in a more recent study.

Male albino mice were given a single oral dose of 180 mg [^{14}C]NTA/kg. The peak ^{14}C level in the blood occurred 1 hr after dosing, demonstrating that NTA was rapidly absorbed from the gastro-intestinal tract. The blood concentration declined rapidly and 4 hr after administration 85% of the radioactivity was no longer in the blood. In mice dosed iv with 45 mg [^{14}C]NTA/kg, the blood concentration was down to one fifth of the peak level after 1 hr. Both after oral and after iv dosing appreciable amounts of radioactivity were found in all tissues after 1 hr but not after 8, 24 or 48 hr.

The highest concentrations of radioactivity were found in the kidney, bladder and bone. Higher concentrations of radioactivity were found in the kidney, heart and skin of the animals dosed iv compared with those dosed orally. Approximately 99% of the oral dose was eliminated within 24 hr, about 96% in the urine and 3–4% via the faeces. The fact that the kidney and bladder were the primary route of excretion for NTA helps to explain the high concentrations of NTA found in these organs 1 hr after dosing. Less than 1% of the iv dose of radioactivity was present in the bile.

The R_F value of the single radioactive band found on thin-layer chromatograms of the urine and faeces was identical with authentic [^{14}C]NTA. The accumulation of NTA in the bones of several other species has previously been reported (*ibid* 1971, **9**, 888) but in the mouse elimination from skeletal tissue was rapid, bone [^{14}C]NTA levels being negligible after 8 hr. The authors conclude that NTA would be unlikely to have any adverse effect on the bones of mice because of its short half-life.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

The fate of trichlorocarbanilide

Birch, C. G., Hiles, R. A., Eichhold, T. H., Jeffcoat, A. R., Handy, R. W., Hill, J. M., Willis, S. L., Hess, T. R. & Wall, M. E. (1978). Biotransformation products of 3,4,4′-trichlorocarbanilide in rat, monkey, and man. *Drug Metab. Dispos.* **6**, 169.

Hiles, R. A. & Birch, C. G. (1978). The absorption, excretion, and biotransformation of 3,4,4′-trichlorocarbanilide in humans. *Drug Metab. Dispos.* **6**, 177.

3,4,4′-Trichlorocarbanilide (TCC) is an antibacterial agent, which has been used in bar soaps and other cleaning products. Dermal studies in guinea-pigs and

man (*Cited in F.C.T.* 1975, **14**, 159) and in rats (Hiles, *Fd Cosmet. Toxicol.* 1977, **15**, 205) indicated that absorption through intact skin was very low and that deposition in the skin was largely limited to the stratum corneum. In the rat study, comparison of elimination routes and tissue distribution after oral, iv and dermal administration of TCC indicated that oral administration, by which a usefully high systemic level could be achieved, was an appropriate route to use for metabolic studies. Oral doses were therefore used for the majority of the metabolic studies now reported.

TCC uniformly labelled with ^{14}C in the monochlorophenyl ring was given orally in a single dose of 0·7 mg/kg to six men and in ten doses of 25·5 mg/kg on consecutive days to an adult male rhesus monkey, and was fed to adult male rats for 5 days at a dietary level providing an intake of 100 mg/kg/day. Other male rhesus monkeys were given [^{14}C]TCC by iv infusion at a rate of 0·18 mg/kg/hr for 10 hr.

Human and monkey urine contained *N*- and *N'*-TCC glucuronides as the major metabolites. In contrast, the rat urine contained a complex mixture of metabolites. Human, monkey and rat plasma contained sulphate and glucuronide conjugates of 2'-, 3'- and 6-hydroxyTCC, and rat plasma also contained conjugates of 2',6-dihydroxyTCC. Monkey bile contained 2'-, 3'- and 6-hydroxyTCC. Since the urine and plasma of man and of rhesus monkeys contained the same major metabolites while the situation in the rat was different, the monkey must be considered on metabolic grounds the species of choice for studies of the toxicity of TCC for man.

In the second paper, describing human experiments with TCC labelled with ^{14}C in the 4'-chlorophenyl ring, in the 3,4-dichlorophenyl ring or in the carbonyl group and administered in corn oil, 70% of an oral dose of 1·9–2·4 μmol [^{14}C]TCC/kg was eliminated in the faeces within 120 hr and 27% was excreted in the urine within 80 hr. Peak plasma concentrations of [^{14}C], equivalent to 3·7 nmol TCC/g, were reached 2·8 hr after dosing. Metabolism of TCC was rapid, but comparison of the findings with the differently labelled compounds indicated that the basic molecule was not split. The major transformation of TCC was by *N*- and *N'*-glucuronidation, as shown in the study mentioned above, or by ring hydroxylation followed by *O*-conjugation with glucuronic or sulphuric acid. The half-life for the major plasma metabolites eliminated into the urine was 2 hr, while that for the 2'-hydroxy- and 6-hydroxyTCC sulphates was 20 hr. The fact that these *o*-hydroxyTCC sulphates are major plasma metabolites in both man and monkey, but are found in the urine of neither species, indicates that bile is an important route for TCC elimination in man as well as in the monkey. This is supported by a demonstration that 54% of an iv dose of [^{14}C]TCC was excreted in human faeces. Thus the appearance of only 27% of a dose of TCC in the urine of orally dosed volunteers was not a true reflection of the degree of absorption in the human gut. Nevertheless the authors consider that a relatively constant fraction of TCC was eliminated rapidly into the urine as *N*-glucuronides, an analytical method for these conjugates in the urine would be useful for estimating total systemic exposure to TCC either in the context of consumer products or the industrial environment.

The cost of home insulation?

Morin, N. C. & Kubinski, H. (1978). Potential toxicity of materials used for home insulation. *Ecotoxic. envir. Safety* **2**, 133.

With the current energy crisis increasing the cost of heating fuels, more people are turning to house insulation in a bid to reduce their fuel bills. In one common method of insulation, wall cavities are filled with a urea-formaldehyde foam. However, it is now reported that the effects of this method of insulation may not be entirely beneficial.

The foam is produced in a foam-forming gun by mixing a solution of urea and formaldehyde (the 'resin') with air and with a mixture of a catalyst and a surfactant (the 'foaming solution'). In addition to these main ingredients, manufacturers also add antifungal and deodorizing agents, fire retardants and other additives. Once the foam has filled the wall cavity, it takes several weeks to dry and cure.

In the study cited above, the way in which the ingredients used for the production of this insulating foam could react with cellular macromolecules was investigated. *Escherichia coli* cells were incubated with [^{32}P]DNA and with either the foam resin or the foaming solution for 60 min at 37°C. With the foaming solution (0·8 or 3% in the incubation medium), the binding of [^{32}P]DNA to the bacterial cells was considerably increased compared with the controls. The foam resin (0·3, 0·6, 3 or 6% in the incubation medium) also increased binding, but only in the presence of mouse-liver extract. Ehrlich ascites cells also bound considerably more [^{32}P]DNA in the presence of the foaming solution (0·3%) than in control incubations, and the binding was further increased by the addition of lysozyme.

Exposure to the foaming solution for 60 min at 37°C caused physico-chemical changes in *E. coli* [^{32}P]DNA. Results of neutral sucrose centrifugation and gel electrophoresis indicated that the apparent molecular weight of the DNA generally decreased, although a few DNA–DNA complexes were formed. No changes in molecular weight were detected when the [^{32}P]DNA was incubated with the foam resin alone, but with the addition of liver extract there was evidence of the formation of complexes between DNA molecules and/or between DNA and the proteins present in the extract. Similar results were obtained when these experiments were repeated using *Cytophaga johnsonii* DNA.

It has been observed that carcinogens decrease the amount of DNA remaining in the aqueous phase during phenol extraction of cellular DNA Following a 60-min exposure of [^{3}H]thymidine-labelled human HeLa cells to the foaming solution there was a dose-related decrease in the amount of DNA recovered in the aqueous phase.

However, although the foam ingredients had effects on DNA, no binding occurred between *E. coli* DNA and *E. coli* cells incubated with aqueous or dimethylsulphoxide extracts of crushed, cured foam. Detailed formulations of the foams were not available and the substances responsible for the effects are not known. Formaldehyde, one of the major ingredients, has mutagenic and DNA-damaging effects in some organisms, but the relevance of these effects to man is

questionable and there is no firm evidence that formaldehyde is carcinogenic in mammals (Cooper, *Fd Cosmet. Toxicol.* 1979, **17**, 300). Apparently, in the USA at present there is little protection from exposure to the foam during its application for either the applicators or the general public. It is suggested that steps should be taken to minimize human contact with the starting materials and that further toxicological tests on the substances should be carried out without delay.

[A manuscript stated to be in preparation promises to provide some useful data for the verification of the procedures used in this study Meanwhile, as with other short-term tests in a relatively early stage of development, the relevance of these findings remains questionable. Comparison of these results with those of other short-term predictive tests, including an Ames test, would be of interest and might establish a need for more elaborate studies. Another problem in this connection would be the need for a clearer identification of the raw materials and their components.]

Fd Cosmet. Toxicol. Vol. 18, pp. 111 to 112
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MEETING ANNOUNCEMENTS

BIOMATERIALS CONGRESS IN AUSTRIA

The First World Biomaterials Congress, to be held in Baden, near Vienna, on 8–12 April 1980, is being organized by the European Society for Biomaterials, the Society for Biomaterials (USA) and the Biomaterials Group of the Biological Engineering Society (UK). The conference will review the current situation and future trends in biomaterials science in such areas as tissue and blood behaviour, tissue adhesives, reconstructive surgery, ophthalmology, artificial organs and drug-delivery systems. Further information may be obtained from: World Biomaterials Congress Secretariat, Mrs. E. Maurer, Medical Academy of Vienna, Alser Strasse 4, A-1090 Vienna, Austria (telephone no. (0222) 42 71 65).

POSTGRADUATE SCHOOL ON TOXICITY TESTING

A postgraduate school on various aspects of toxicological testing methods is being organized jointly by the Department of Pharmacy, Chelsea College, and the Pharmaceutical Society of Great Britain. The school will run from 14 to 18 April 1980. Mornings will be devoted to lectures and discussions covering the main aspects of toxicity testing, and in the afternoons there will be further lectures and a supporting programme of demonstrations and seminars. Information on the school may be obtained from Mr. R. E. Marshall, School Secretary, Department of Pharmaceutical Sciences, The Pharmaceutical Society of Great Britain, 1 Lambeth Street, London SE1 7JN (telephone no. 01–735 9141, ext. 287).

FOODBORNE INFECTIONS

The World Congress on Foodborne Infections and Intoxications will be held in the International Congress Centre, Berlin (West), from 29 June to 3 July 1980. The Congress is being organized by the Institute of Veterinary Medicine, Robert von Ostertag-Institute, D-1000 Berlin 33, Thielallee 88–92 (telephone no. (030) 83 08–1; Telex 01 84 016). The Secretary General is Dr. K. Gerigk.

INTERNATIONAL CONGRESS ON TOXICOLOGY

The final announcement and detailed programme for the Second International Congress on Toxicology (Fd Cosmet. Toxicol. 1979, **17**, 321) has now been issued. Readers are reminded that the congress, which will be held in the Congress Centre, Brussels, from 6 to 11 July 1980, will be concerned with mechanisms of toxicity and hazard evaluation. Individual plenary sessions will focus on mechanisms of neurotoxicity, short-term tests for predicting long-term effects, early changes in chemical carcinogenesis, long-term exposure to occupational intoxicants, clinical toxicology, and the legislative, scientific and socioeconomic considerations underlying the toxicological testing of new chemicals.

Further information may be obtained from SdR Associated, Avenue des Abeilles 16, 1050 Brussels, Belgium (telephone no. (02) 647 8780).

CHEMICAL MECHANISMS AND BIOLOGICAL EFFECTS

The Second International Symposium on Biological Reactive Intermediates: Chemical Mechanisms and Biological Effects will be held at the University of Surrey, Guildford, on 14–17 July 1980. The programme will be concerned with the formation and disposition of biological reactive intermediates, with oxygen activation in xenobiotic metabolism and specifically with the reactive intermediates associated with several major groups of chemical compounds.

Further details may be obtained from Prof. D. V. Parke, Department of Biochemistry, University of Surrey, Guildford, Surrey G42 5XH.

THE RAT ELECTROCARDIOGRAM IN TOXICOLOGY

An International Workshop on the Rat Electrocardiogram in Acute and Chronic Pharmacology and Toxicology will be held in Hannover on 14 and 15 July 1980.

Further information may be obtained from Dr. R. Budden, Abteilung Spezielle Pharmakologie, Sparte Pharma, Kali-Chemie AG, Hannover, Federal Republic of Germany.

CANCER PREVENTION AND DETECTION

The Royal Society of Medicine and the International Study Group for the Detection and Prevention of Cancer are sponsors of the Fourth International Symposium on the Prevention and Detection of Cancer, to be held at the Wembley Conference Centre, London, from 26 to 31 July 1980. The scientific programme will deal with primary prevention through the identification of environmental, occupational, genetic and other factors constituting a cancer risk, as well as with secondary prevention through the early detection of neoplasms. A range of sessions, panels, workshops and discussions will be arranged to review specific topics.

Requests for further information should be directed to: The Conference Centre, 43 Charles Street, Mayfair, London W1X 7PB (telephone no. 01–499 1101).

INTERNATIONAL COSMETICS MEETING

The 11th International IFSCC Congress will be held at the Hotel Excelsior, Venice Lido, Italy, from 23 to 26 September 1980. The main part of the Congress Programme will be scientific conference which, under the title "Cosmetics: Research and Technology" will consider the efficacy and safety of cosmetics (two sessions), analytical control methods, the stability of cosmetic products and microbiological controls, manufacturing techniques and good manufacturing practice, and the biology of skin and hair.

The Congress is being organized by the Societa Italiana dei Chimici Cosmetologi. Registration forms and a provisional programme are available from: Mrs. M. Fadini, The Organizing Secretariat, 11th IFSCC International Congress, c/o Studio MGR, Piazza Sant 'Ambrogio 16, 20123 Milan, Italy.

IUPAC SYMPOSIUM ON TOXICITY MECHANISMS

The International Union of Pure and Applied Chemistry is sponsoring a symposium entitled "Chemical Indices and Mechanisms of Organ-directed Toxicity", to be held in the Congress Centre, Barcelona, Spain, on 4–7 March 1981. Attention will be focused on drug-related toxic effects, but relevant studies of environmental and occupational xenobiotics will also be considered. Sessions covering both clinical and experimental aspects will survey, in depth, each of the principal target organs.

Requests for further details of the symposium should be sent to: Symposium on Chemical Mechanisms of Organ-directed Toxicity, 142–144 Oxford Road, Cowley, Oxford OX4 2DZ.

CHEMICAL MUTAGENESIS SYMPOSIUM IN CANADA

An International Symposium on "Chemical Mutagenesis: Human Population Monitoring and Genetic Risk Assessment" will be held in Ottawa on 14–16 October 1980, under the auspices of the Department of National Health and Welfare, Canada. Sponsoring organizations include the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC) and the Genetics Society of Canada.

The Symposium will review four major aspects of the subject, namely current problems in identifying and studying human populations exposed to known or suspected mutagens or carcinogens, methods of mutagen detection, assessment of genetic risk, and strategies for future development. A poster session, limited to 50 selected presentations, will be included in the programme. The number of participants will be limited to 400 and the registration fee is approximately US $100.00. Further details may be obtained from Dr. K. C. Bora, Environmental Health Directorate, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2.

Fd Cosmet. Toxicol. Vol. 18, p. 113
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FORTHCOMING PAPERS

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

Mutagenicity of ethylene oxide and propylene oxide and of the glycols and halohydrins formed from them during the fumigation of foodstuffs. By E. H. Pfeiffer and H. Dunkelberg.

Effect of malondialdehyde on nitrosamine formation. By T. Kurechi, K. Kikugawa and M. Ozawa.

Effet comparé de l'acide linoléique et de l'acide linoléique peroxydé sur l'activité d'enzymes de l'entérocyte isolé de l'intestin de rat. Par C. Luong Dinh, R. A. Boigegrain, S. Mitjavila et R. Derache.

Effect of the plant growth regulator, 2-chloroethylphosphonic acid, on spontaneous and chemically-induced lung tumorigenesis in strain A mice. By J. C. Theiss and M. B. Shimkin.

Stable lead, ^{210}Pb and ^{210}Po in the liver and kidneys of cattle—II. Animals from an area near an abandoned lead mine. By K. Bunzl, W. Kracke and W. Kreuzer.

The protective potency of marine animal meat against the neurotoxicity of methylmercury: its relationship with the organ distribution of mercury and selenium in the rat. By G. Ohi, S. Nishigaki, H. Seki, Y. Tamura, T. Maki, K. Minowa, Y. Shimamura, I. Mizoguchi, Y. Inaba, Y. Takizawa and Y. Kawanishi.

Nitrosamines in new motor-cars. By D. P. Rounbehler, J. Reisch and D. H. Fine.

Transfer of polychlorinated dibenzofurans to the foetuses and offspring of mice. By J. Nagayama, S. Tokudome, M. Kuratsune and Y. Masuda.

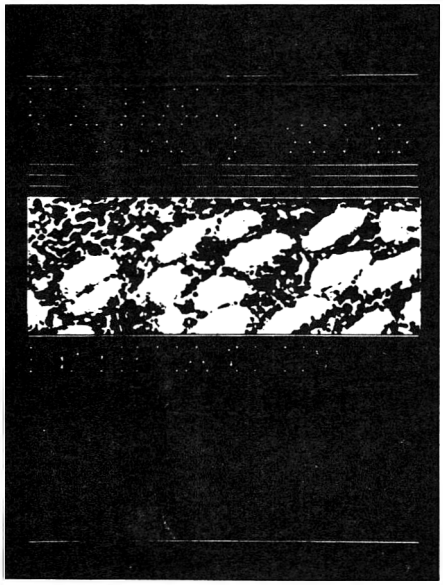
Toxicology of glucosinolates, related compounds (nitriles, *R*-goitrin, isothiocyanates) and vitamine U found in *Cruciferae*. By K. Nishie and M. E. Daxenbichler.

Patulin mycotoxicosis in the Syrian hamster. By E. R. McKinley and W. W. Carlton.

Patulin mycotoxicosis in Swiss ICR mice. By E. R. McKinley and W. W. Carlton.

Teratogenicity study of dicetyldimethylammonium chloride in mice. By K. Inoue and M. Takamuku.

European Journal of Cancer



Editor: **H J Tagnon** *Institut Jules Bordet, Brussels, (Belgium)*

The European Journal of Cancer was founded primarily to encourage collaboration among scientists and clinical investigators in the broad field of cancer research. Such collaboration has already found its expression in the creation of several European work groups, such as the European Organization for Research on Treatment of Cancer, with its many co-operative sub-groups (mammary cancer, lung cancer, prostatic cancer, children's tumors, head and neck cancer, clinical radiobiology, etc.). There was a need for exchange of ideas and information among workers belonging to all scientific disciplines concerned with cancer. The foundation of the European Journal of Cancer represents the inevitable outcome of the fruitful collaboration which has arisen spontaneously among the countries of Europe.

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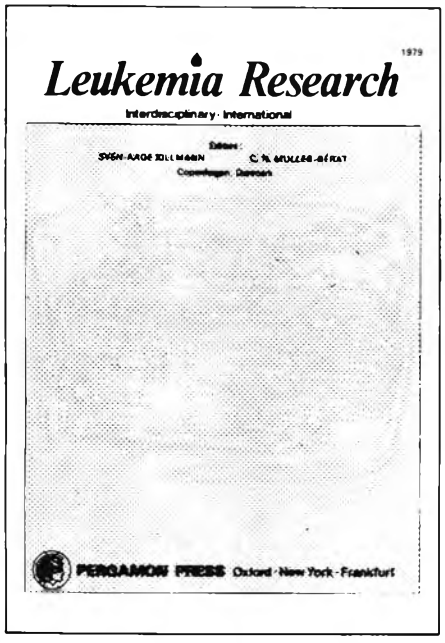
Leukemia Research



The International Interdisciplinary Journal for the Rapid Publication of Original Research Communications on All Aspects of Leukemia

Editors: **Sven-Aage Killmann,** *University Hospital of Copenhagen, Denmark* and **C N Muller-Bérat,** *Statens Seruminstitut, Copenhagen, Denmark*

Leukemia Research aims to promote interdisciplinary confrontation of high quality publications pertinent to leukemia and closely related diseases. Consequently, there will be no restriction as to the scientific field of authors, the methods, the material (human as well as animal), the approach (clinical, biological or dealing with pure basic sciences), as long as the data are experimental and of high standard. Contributions which do not deal directly with leukemia, but deal with normal blood and tissue as well as with cell biology at large are highly welcome in so far as they carry a message of general interest for the understanding of the deranged mechanisms of the biology of the leukemic cell. Such would be papers dealing with differentiation or molecular biology of growth control.

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[*Contents continued*]

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.

Instructions to Authors

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor. All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced*. At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal [abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*, 4th Ed., Butterworth & Co. (Publishers) Ltd, London], volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation—1, Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum*. 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b, etc. following the citation of the year:

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Footnotes. These, as distinct from literature references, should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § ‖ ¶ in that order.

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Tables and Figures. These should be constructed so as to be intelligible without reference to the text, each table and column being provided with a heading. The same information should not be reproduced in both tables and figures.

Chemical Nomenclature. The fundamental principles of organic and inorganic chemical nomenclature are laid down in the I.U.P.A.C. 1957 Rules (Butterworths Scientific Publications, London, 1958, 1959). These are given in *Handbook for Chemical Society Authors* (1961), pp. 16–163.

Other Nomenclature, Symbols and Abbreviations. In general, authors should follow the recommendations published in the *Handbook for Chemical Society Authors* (1961), p. 164 and in the *I.U.P.A.C. Information Bulletin*, No. 13, p. 64, Appendix B (1961). In the title and summary, abbreviations should be avoided; in the Introduction, Results and Discussion they should be used sparingly.

Page Proofs. These will be sent to the first-named author for correction.

Reprints. Reprints and copies of the issue in which the article appears can be ordered on the form accompanying proofs.

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