Volume 20 Number 4 August 1982

Author and subject indexes to Volume 19 (1981) included loose in this issue

Food and Chemical Toxicology

An International Journal published for the British Industrial Biological Research Association

(formerly Food and Cosmetics Toxicology)

RESEARCH SECTION

Continued on inside back cover

ISSN 0278-6915

FCTOD7 20(4) 351–498 (1982)

Pergamon Press OXFORD NEW YORK TORONTO SYDNEY PARIS FRANKFURT

FOOD AND CHEMICAL TOXICOLOGY

An International Journal published for the British Industrial Biological Research Association

Publishing Office

Journals Production Unit, Hennock Road, Marsh Barton, Exeter EX2 8RP, England
(*Tel.* Exeter (0392) 51558; *Telex* 42749)

Subscription enquiries and Advertising Offices

North America: Pergamon Press Inc., Fairview Park, Elmsford, NY 10523, USA
Rest of the World: Pergamon Press Ltd, Headington Hill Hall, Oxford OX3 0BW, England

Published bi-monthly

Annual Subscription Rates (1982)

For Libraries, University Departments, Government Laboratories, Industrial and all other multiple-reader institutions US $260.00 per annum (including postage and insurance), 2-year subscription rate $494.00.
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PERGAMON PRESS LIMITED

HEADINGTON HILL HALL, OXFORD OX3 0BW, ENGLAND

MAXWELL HOUSE, FAIRVIEW PARK, ELMSFORD, NY 10523, USA

Fd Chem. Toxic. Vol. 20, p. i
Pergamon Press Ltd 1982. Printed in Great Britain

INFORMATION SECTION

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Fd Chem. Toxic. Vol. 20, pp. 351 to 356, 1982
Printed in Great Britain.

0278-6915/82/040351-06$03.00/0

Research Section

THE METABOLIC DISPOSITION OF ^{14}C-LABELLED CARMOISINE IN THE RAT AFTER ORAL AND INTRAVENOUS ADMINISTRATION

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(*Received 26 October 1981; revision received 30 December 1981*)

Abstract—The absorption, distribution and excretion of the red azo dye carmoisine (Ext. D & C No. 10) was studied in male rats. [^{14}C]Carmoisine was administered in a dose of 200 mg/kg (25 μCi) by gavage or in the same dose (200 mg/kg; 3 μCi) by intravenous injection, and radioactivity was measured in blood, tissue, faeces and urine at different times after dosing. After oral administration of the dye, no radioactivity was detected in the brain, adipose tissue, muscle, testes, spleen or lung, and recovery of the administered activity in faeces and urine was almost complete by 32 hr. The radioactivity profile of the blood indicated rapid but poor absorption of [^{14}C]carmoisine, a maximum radioactivity content corresponding to 0·01% of the dose per ml of blood being reached within 10 min. The decay curve for ^{14}C radioactivity in the blood after iv injection of [^{14}C]carmoisine indicated rapid distribution to the tissues and could be described in terms of a two-compartment mathematical model. The highest levels of radioactivity occurred in the gastro-intestinal tract and liver after the injection but after 24 hr no radioactivity was detectable in these or other tissues. All the radioactivity was recovered in the faeces and urine in the 24 hr following iv injection, the 79% of the dose present in faeces indicating active excretion of the dye and its metabolites in the bile and poor reabsorption from the intestine. The bioavailability of [^{14}C]carmoisine, calculated from the blood-radioactivity curves after oral and iv administration, was less than 10%.

INTRODUCTION

Water-soluble sulphonated azo dyes are used extensively as colourings for food, drugs and cosmetics. With the increasing awareness of possible health hazards associated with such use, more attention has been focused recently on the biological activity of the dyes. During the past decade several azo dyes, notably Ponceau 4R and amaranth (FD & C Red No. 2), have been banned as food additives in some countries because of their association with toxic effects such as liver injury and tumour formation in experimental animals (Adrianova, 1970; Baigusheva, 1968; Grice, Mannell & Allmark, 1961; Hansen, Davis, Fitzhugh & Nelson, 1963).

The absence of data on the pharmacokinetics and metabolism of azo dyes and the potential formation of toxic metabolites by reductive cleavage of the azo linkage has caused many international agencies to require metabolic and toxicokinetic studies in different species. Data have recently been published on the metabolic disposition of ^{14}C-labelled amaranth in the rat (Ruddick, Craig, Stavric *et al.* 1979) and of [^{14}C]carmoisine in the mouse (Galli, Marinovich & Costa, 1981a). Furthermore, the British Industrial Biological Research Association is carrying out a series of metabolic studies on food colourings, in the rat, mouse and guinea-pig, as part of its safety evaluation programme.

Carmoisine (azorubine; Ext. D & C Red No. 10; C.I. (1965) No. 14720; E122) is a red azo dye permitted for use in food in the EEC. No toxic effects were found following administration of carmoisine in single oral doses of 8 g/kg to the mouse and 10 g/kg to the rat (Gaunt, Farmer, Grasso & Gangolli, 1967), in doses up to 250 mg/kg/day in a short-term feeding study in rats (Gaunt *et al.* 1967), in doses up to 350 mg/kg/day in a long-term study in mice (Mason, Gaunt, Butterworth *et al.* 1974) or at dietary levels of up to 2% in a study of reproductive toxicity and in a 1-yr feeding study (including *in utero* exposure) in rats (Holmes, Pritchard & Kirschman, 1978a,b).

In its twenty-second report, the Joint FAO/WHO Expert Committee on Food Additives (1978) increased the admissible daily intake (ADI) for carmoisine from 0·5 to 1·25 mg/kg body weight on the basis of the 90-day feeding study in rats, but did not change the 'temporary' classification because long-term studies in the rat were considered inadequate. It was requested that the results of toxicokinetic and metabolism studies of the dye in different species, as well as the results of extra feeding studies, should be presented to the Committee by 1982 so that a full ADI could be allocated. For this reason we have studied the absorption, fate and excretion of carmoisine in rats following oral and intravenous administration and compared the findings with those of a similar study previously carried out in mice (Galli *et al.* 1981a).

EXPERIMENTAL

Test material. [1,4,5,8,1′,4′,5′,8′-^{14}C]Carmoisine (Fig. 1), 99% radiochemically pure and with a specific activity of 5 mCi/mmol was purchased from the Radiochemical Centre Ltd, Amersham, Bucks, UK, and was generously supplied by Davide Campari S.p.A., Milan. Unlabelled carmoisine, complying fully

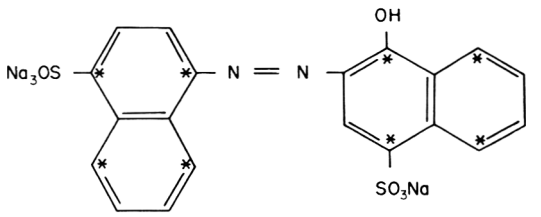

Fig. 1. Chemical structure of the [^{14}C]carmoisine used, with asterisk locating the ^{14}C label.

with the EEC and FDA purity requirements and having a dye content greater than 87% was obtained from B.V. Nederlanse Kleurstoff-Industrie, Amersfoort, The Netherlands, and was used to adjust the dose of [^{14}C]carmoisine to the desired specific activity. The chief impurities were sodium chloride (6%) and volatile matter 6·2%).

Animals and treatment. Male Sprague–Dawley rats (Charles River Inc., Calco, Como) weighing 150–200 g were used in all the experiments. After a 12-hr fast, each rat was dosed with [^{14}C]carmoisine either orally (200 mg/kg body weight: 25 μCi) or by injection into the tail vein (200 mg/kg body weight: 3 μCi), the dye being dissolved in distilled water to a final volume of 0·3 and 0·25 ml, respectively. The animals were housed individually in metabolic cages (Techniplast, Buguggiate, Varese) and had free access to a standard diet (4 RF 21, from Charles River Inc.) and water after dosing.

Groups of rats were killed by decapitation 5, 10, and 30 min and 1, 2, 4, 8, 16, 32, 64 and 96 hr after carmoisine administration and blood was collected in heparinized tubes. The gastro-intestinal tract, liver, testes, lungs, brain, spleen, kidneys, epididymal adipose tissue and muscle were removed. Urine and faeces were collected from the metabolic cage. In all cases the biological samples were frozen on dry ice immediately after removal and were kept at −20°C until used for radioactivity measurement.

Determination of radioactivity. After homogenization, duplicate samples of approximately 10–20 mg of all tissues were solubilized in 1·0 ml Lumasolve (Lumac System AG, Basel, Switzerland) at 50°C for 30 min. Lipoluma (5·0 ml; Lumac System AG) was added to all vials as scintillation liquid. Blood (50-μl aliquots) was added to 0·5 ml of a Lumasolve–isopropanol solution (1:2, v/v) and shaken for 60 min at 50°C, after which 0·5 ml 35% (v/v) H_2O_2 was added dropwise to each vial, the samples were kept for 30 min at room temperature and then 10 ml Lumagel–0·5 N-HCl (9:1, v/v) was added as scintillation liquid. The radioactivity in urine was determined by adding 10 ml Lumagel (Lumac System AG) to a 0·1-ml aliquot. Faeces were suspended in 4 vols water, allowed to swell over the next 2 days and periodically stirred. To 50 μl of the mixture, 0·5 ml Lumasolve–isopropanol (1:2, v/v) was added and the samples were digested overnight at 50°C with shaking. Cooled samples were bleached with 0·5 ml 35% (v/v) H_2O_2, and 15 ml Lumagel-0·5 N-HCl (9:1, v/v) was added before counting. Levels of ^{14}C activity in the samples were determined with a Packard Tri-Carb 3255 scintillation counter.

Efficiency of the counter was determined for each sample using external standard channel ratios and referring to appropriate quenching curves. Tissues of control (untreated) rats were processed in the same manner and the counts generated were considered as background.

Analysis of data. Plasma concentration–time curves after intravenous administration of [^{14}C]carmoisine were determined after calculating the log plasma concentration as a function of time Cp = f(t) regression lines of the β phase (elimination phase) and of the residual of the α-phase distribution phase) according to a two-component open model (Lintz, Berger, Aenishaenslin *et al.* 1974). After plotting the blood concentration data the hybrid constants A, α, B and β were computed:

A = intercept at time zero of the first order plot of slope $-\alpha$ obtained from the 'feathered' plot of ln C against time, where B and β are obtained and then the residuals are plotted to obtain A and α.

α = apparent first order rate constant for distribution of the drug in the body.

B = intercept at time zero of the first order plot of slope $-\beta$ obtained from the plot of ln C against time.

β = apparent first order rate constant for elimination of the drug from the body.

From these constants, pharmacokinetic parameters were determined according to the following equations:

Total area under plasma concentration curve—

$$AUC_{iv} = \frac{A}{\alpha} + \frac{B}{\beta}. \quad \ldots(1)$$

Half life—0·693/β or α.

Bioavailability after oral administration of [^{14}C]-carmoisine was determined according to equation 2—

$$Bioav = \frac{AUC_{po} \cdot dose_{iv}}{AUC_{iv} \cdot dose_{po}} \quad \ldots(2)$$

the AUC being estimated by the trapezoidal rule.

RESULTS

After oral and iv administration of the dye, no significant difference was found between treated and control animals in body and organ weights or food and water consumption.

Within 5 min of oral administration of the dye, the blood levels of radioactivity were significantly higher than the blank values, indicating a rapid absorption of carmoisine. The highest level of radioactivity, corresponding to 0·01% of the orally administered dose per ml of blood, was reached within 10 min and then slowly declined, no ^{14}C activity being detected at 32 hr (Fig. 2). Radioactivity was not detected in the brain after any interval, indicating that neither the azo dye nor its metabolites crossed the blood–brain barrier. Among the other tissues analysed, the lungs, testes, spleen, epididymal adipose tissue and muscle showed no levels of radioactivity significantly above the blank values. In the gastro-intestinal tract and

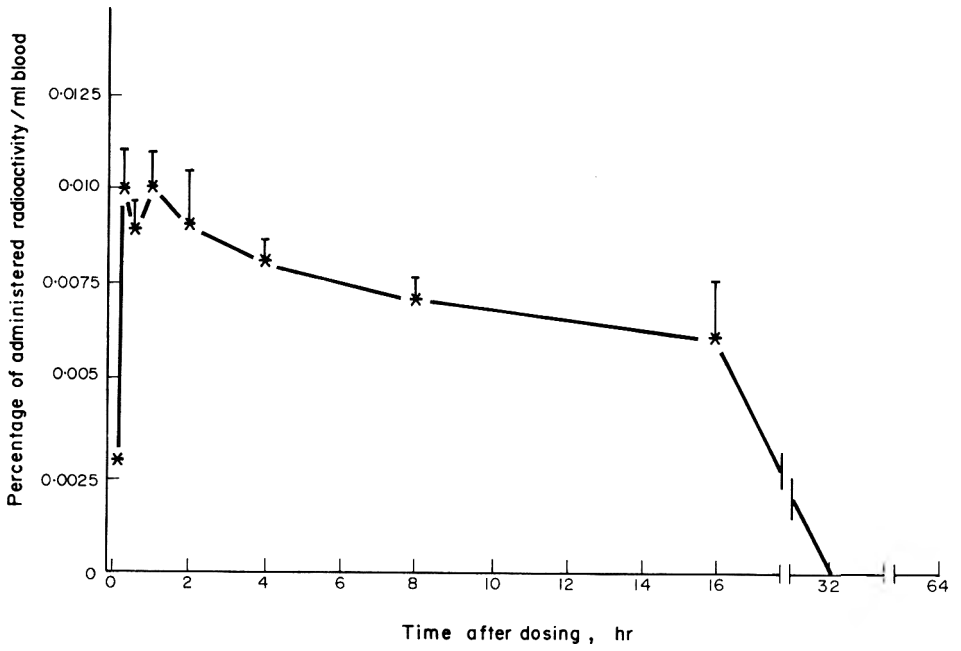

Fig. 2. Disappearance of ^{14}C activity from the blood of rats following oral administration of a single dose of [^{14}C]carmoisine (200 mg/kg; 25 μCi). Each point represents the mean ± SEM for groups of at least three rats.

liver, radioactivity levels were very well correlated with the blood values at all times after oral administration (Table 1).

The rate of disappearance from tissues and blood suggests that no accumulation of the dye and/or its metabolites occurs. Recovery in the urine and faeces accounted for 50% of the oral dose of ^{14}C activity in the first 16 hr after dosing and for as much as 90% over a 32-hr period (Table 2).

Levels of radioactivity in the tissues after iv administration of [^{14}C]carmoisine are shown in Table 3. As in the oral study, the distribution of radioactivity in the tissues was homogeneous and no concentration occurred in any tissue. No radioactivity was detected in the liver, lungs, spleen, muscle, brain, epididymal adipose tissue and testes 24 hr after injection, and at this sampling time only the kidneys and the gastro-intestinal tract were found to have a measurable

Table 1. *Distribution of radioactivity in the gastro-intestinal tract, liver and kidney of rats following administration of a single oral dose of [^{14}C]carmoisine (200 mg/kg; 25 μCi)*

Time after dosing	No. of rats	^{14}C activity (dpm × 10^4/g)		
		Gastro-intestinal tract	Liver	Kidney
5 min	3	305 ± 10	0·16 ± 0·03	0·18 ± 0·03
10 min	3	333 ± 25	0·52 ± 0·05	0·68 ± 0·02
30 min	3	274 ± 27	0·61 ± 0·25	0·44 ± 0·17
1 hr	3	297 ± 16	0·69 ± 0·09	0·71 ± 0·03
2 hr	3	303 ± 16	0·50 ± 0·10	1·15 ± 0·31
4 hr	6	286 ± 28	0·54 ± 0·08	1·76 ± 0·63
8 hr	3	182 ± 15	0·40 ± 0·07	1·62 ± 0·38
16 hr	3	129 ± 22	0·36 ± 0·09	0·99 ± 0·11
32 hr	6	ND	ND	ND
64 hr	3	ND	ND	ND
96 hr	3	ND	ND	ND

ND = Not detected

Values are means ± SEM for the numbers of animals indicated. Radioactivity measurements for each tissue sample were made in duplicate.

Values were not significantly different from those for the corresponding tissue homogenates from control animals; 46·46 × 10^4 dpm corresponds to 1% of the administered radioactivity.

Table 2. *Excretion of radioactivity in the faeces and urine of rats after administration of a single oral dose of [^{14}C]carmoisine (200 mg/kg; 25 μCi)*

Time after dosing (hr)	No. of rats	Recovery of administered radioactivity (%)			
		In faeces	In urine	In cage wash	Total
4	6	ND	0·8 ± 0·1	0·2	1
8	3	30·0 ± 6·6	3·7 ± 0·1	0·3	34
16	3	41·2 ± 13·3	9·6 ± 3·8	0·9	52
32	6	81·6 ± 6·0	8·3 ± 1·0	0·4	90
64	3	80·6 ± 10·7	16·9 ± 1·9	0·4	98
96	3	73·1 ± 2·4	13·3 ± 0·02	0·3	87

ND = Not detected

Values are means ± SEM for the numbers of animals indicated. Radioactivity measurements for each sample were made in duplicate.

amount of radioactivity (respectively 0·09 and 0·02% of the dose of ^{14}C activity per g tissue; Table 3). Table 4 shows that over a 24-hr period iv-administered carmoisine and/or its metabolites were completely eliminated in the faeces (79%) and urine (19%).

The profile of blood levels of radioactivity after iv administration (Fig. 3) shows a rapid distribution of [^{14}C]carmoisine with a half-life ($t_{\frac{1}{2}}$) for the distribution phase of 0·48 hr (Table 5). Half-lives for the elimination phase demonstrate that the compound is rapidly excreted after both oral and iv administration with $t_{\frac{1}{2}}$ equal to 16·7 and 8·2 hr, respectively (Table 5). The bioavailability, calculated from equation 2, was found to be 9·4% (Table 5).

DISCUSSION

Our results clearly indicate that [^{14}C]carmoisine administered orally to male rats is poorly absorbed, since the maximum level of radioactivity found in the blood corresponded to only 0·01% (per ml) of the administered dose. Distribution of the radioactivity was homogeneous and carmoisine and its metabolites did not appear to accumulate in any of the tissues analysed.

The bioavailability, i.e. the amount of azo dye and/or its metabolites available for potential interaction with endogenous substrates, was calculated from the blood radioactivity curves after oral and iv administration and was found to be only 9·4%.

Both the kinetic profile of radioactivity and tissue distribution of radioactivity after oral administration of [^{14}C]carmoisine are similar to those found when the dye was administered orally to pregnant rats (Galli, Marinovich, Costa & Giavini, 1981b). A comparison of the absorption, distribution and excretion of [^{14}C]carmoisine in rats with that in mice (Galli *et al.* 1981a) indicates that the toxicokinetic profile is not identical in the two species. Absorption efficiency in the rat is about 10% of that in mice and absorption and excretion are much faster in the rat (Galli *et al.* 1981a).

The finding that, after iv injection, levels of radioactivity in the gastro-intestinal tract slowly increase (Table 3) and excretion of radioactivity (Table 4) is higher in the faeces (80%) than in the urine (20%) is in agreement with previous data showing that carmoisine and similar azo dyes administered by iv injection are actively excreted in the bile both in the rats (Ryan & Wright, 1961) and in mice (Galli *et al.* 1981a). It seems likely, therefore, that carmoisine and/or its metabolites are poorly reabsorbed from the intestine, as has been shown for other azo dyes like amaranth (Ruddick *et al.* 1979) and Brown HT (J. C. Phillips, D.

Table 3. *Distribution of radioactivity in tissues of rats after iv injection of a single dose of [^{14}C]carmoisine (200 mg/kg; 3 μCi)*

Tissue	Time after dosing...	^{14}C activity (dpm × 10^4/g)					
		5 min	10 min	30 min	1 hr	2 hr	24 hr
Gastro-intestinal tract		1·6 ± 0·14	3·4 ± 0·07	11·5 ± 1·2	17·2 ± 1·2	24·2 ± 2·3	0·1 ± 0·03
Liver		10·1 ± 0·7	10·5 ± 0·3	5·4 ± 0·3	2·1 ± 0·2	1·1 ± 0·1	ND
Kidney		6·2 ± 0·6	6·4 ± 1·3	1·7 ± 0·2	1·9 ± 0·5	1·2 ± 0·1	0·5 ± 0·04
Lung		6·0 ± 0·2	5·4 ± 0·9	2·6 ± 0·2	0·8 ± 0·06	0·5 ± 0·08	ND
Spleen		1·7 ± 0·006	1·4 ± 0·18	0·4 ± 0·06	0·2 ± 0·03	0·1 ± 0·04	ND
Brain		0·6 ± 0·07	0·4 ± 0·06	0·2 ± 0·05	0·1 ± 0·02	0·08 ± 0·01	ND
Testes		0·6 ± 0·06	0·9 ± 0·06	0·8 ± 0·08	0·7 ± 0·1	0·4 ± 0·03	ND
Muscle		1·7 ± 0·03	1·5 ± 0·07	0·7 ± 0·05	0·3 ± 0·01	0·1 ± 0·006	ND
Adipose tissue		1·4 ± 0·2	1·6 ± 0·05	0·6 ± 0·08	0·3 ± 0·07	0·1 ± 0·03	ND

ND = Not detected

Values are means ± SEM for groups of three animals. Radioactivity measurements for each tissue sample were made in duplicate.

Values were not significantly different from those for the corresponding tissue homogenates from control animals; 5·58 ± 10^4 dpm corresponds to 1% of the administered radioactivity.

Table 4. *Excretion of radioactivity in the faeces and urine of rats following iv injection of a single dose of [^{14}C]carmoisine (200 mg/kg; 3 μCi)*

Time after dosing (hr)	No. of rats	Recovery of administered radioactivity (%)		
		In faeces	In urine	Total
2	2	ND	9	9
4	3	ND	12 ± 0·3	12
8	3	37 ± 0·3	16 ± 1·2	53
24	3	79 ± 0·5	19 ± 1·8	98
48	3	84 ± 0·9	18 ± 0·7	102

ND = Not detected

Values are means ± SEM for the numbers of animals indicated. Radioactivity measurements for each sample were made in duplicate.

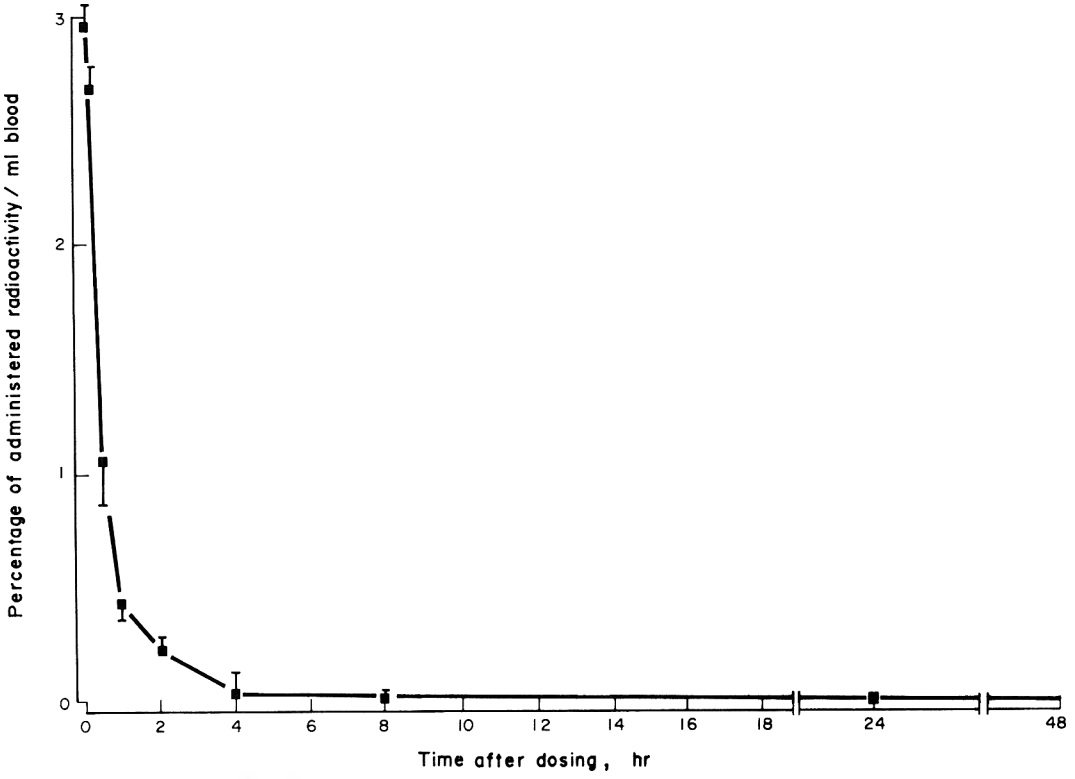

Fig. 3. Disappearance of ^{14}C activity from the blood of rats given a single iv injection of [^{14}C]carmoisine (200 mg/kg; 3 μCi). Each point is the mean ± SEM for groups of three animals.

Table 5. *Disposition constants for [^{14}C]carmoisine following oral and iv administration in a dose of 200 mg/kg*

Constant	Intravenous route	Oral route
A (μg/ml)	701	—
α (min^{-1})	0·023	—
$t_{\frac{1}{2}}$ (hr)	0·48	—
B (μg/ml)	17·0	2·8
β (min^{-1})	0·00141	0·00069
$t_{\frac{1}{2}}$ (hr)	8·19	16·69
AUC (μg.min/ml)	42535	3988

The bioavailability after oral administration, calculated from (AUC_{po} × $dose_{iv}$/AUC_{iv} × $dose_{po}$) × 100, is 9·4%.

Mendis, S. D. Gangolli & I. F. Gaunt, unpublished data, 1981) and Ponceau 4R (Phillips, Bex & Gaunt, 1982) in different species.

The toxicokinetic profile of naphthionic acid, a major metabolite of amaranth, Brown HT and Ponceau 4R has many similarities with those of the parent compounds (Pritchard, Holmes & Kirschman, 1976; Radomski & Mellinger, 1962). Experiments in progress in our laboratory point to naphthionic acid as one of the carmoisine metabolites present in the faeces and urine of rats (Galli & Costa, 1980). Separation of metabolites by high-pressure liquid chromatography coupled with a radioactivity monitor provided evidence of the presence of four other compounds whose structure is under investigation.

Acknowledgements—The authors thank Mrs Aurora Maccalli Rossoni and Miss Lucia Maetzke for secretarial assistance. This study was supported by a grant from Davide Compari S.p.A., Milan.

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Fd Chem. Toxic. Vol. 20, pp. 357 to 363, 1982
Printed in Great Britain.

0278-6915/82/040357-07$03.00/0

MUTAGENS FROM THE COOKING OF FOOD. II. SURVEY BY AMES/SALMONELLA TEST OF MUTAGEN FORMATION IN THE MAJOR PROTEIN-RICH FOODS OF THE AMERICAN DIET

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(*Received 25 August 1981*)

Abstract—The formation of mutagens in the major cooked protein-rich foods in the US diet was studied in the Ames *Salmonella typhimurium* test. The nine protein-rich foods most commonly eaten in the USA—ground beef, beef steak, eggs, pork chops, fried chicken, pot-roasted beef, ham, roast beef and bacon—were examined for their mutagenicity towards *S. typhimurium* TA1538 after normal 'household' cooking (deep frying, griddle/pan frying, baking/roasting, broiling, stewing, braising or boiling at 100–475°C). Well-done fried ground beef, beef steak, ham, pork chops and bacon showed significant mutagen formation. For chicken and beef steak high-temperature broiling produced the most mutagenicity, followed by baking/roasting and frying. Stewing, braising and deep frying produced little mutagen. Eggs and egg products produced mutagens only after cooking at high temperatures (the yolk to a greater extent than the white). Commercially cooked hamburgers showed a wide range of mutagenic activity. We conclude that mutagen formation following cooking of protein-containing foods is a complex function of food type, cooking time and cooking temperature. It seems clear that all the major protein-rich foods if cooked to a well-done state on the griddle (eggs only at temperatures above 225°C) or by broiling will contain mutagens detectable by the Ames/Salmonella assay. This survey is a step towards determining whether any human health hazard results from cooking protein-rich foods. Further testing in both short- and long-term genotoxicity bioassays and carcinogenesis assays are needed before any human risk extrapolations can be made.

INTRODUCTION

The occurrence of mutagenic substances in certain heated foods and food components is now well documented. Proteins and several amino acids yield highly mutagenic substances when pyrolysed at temperatures above 300°C (Matsumoto, Yoshida, Mizusaki & Okamoto, 1977; Matsumoto, Yoshida, Mizusaki & Okamoto, 1978; Nagao, Honda, Seino *et al.* 1977). Similar temperatures were reported to be necessary to generate appreciable mutagenicity in pyrolysed food samples (Uyeta, Kanada, Mazaki & Taue, 1978). Under pyrolytic conditions, carbohydrate and lipid components and foods rich in them tend to exhibit less mutagenic activity than samples rich in proteins or amino acids. Evidence compiled by several research groups indicates that mutagenic activity is also induced in certain foods cooked under conditions less severe than required for pyrolysis (Commoner, Vithayathil, Dolara *et al.* 1978; Pariza, Ashoor, Chu & Lund, 1979; Spingarn & Weisburger, 1979; Felton, Healy, Stuermer *et al.* 1981). Mutagenic activity is present in fried and broiled protein-rich food (beef) at a higher level than in cooked carbohydrate-rich food (Spingarn, Slocum & Weisburger, 1980). Broiled fish samples also contain mutagen (Kasai, Nishimura, Nagao *et al.* 1979). In addition, extended boiling of beef stock results in mutagen formation (Commoner *et al.* 1978).

The purpose of our study was to survey cooked protein-rich foods for mutagenic activity. The major protein-rich foods in the American diet were identified by computer analysis of studies by the US Department of Agriculture (USDA) survey from 1964–1966 and the US Department of HEW (HANES) survey from 1971–1975 (Plumlee, Bjeldanes & Hatch, 1981). The study included normal cooking times and temperatures as well as more severe conditions. Mutagen formation during different cooking methods is compared for beef, pork, chicken and eggs. Finally, we give an estimate of the daily mutagen intake from cooked protein-rich foods in the average American diet, when the foods are 'well-done'.

EXPERIMENTAL

General cooking procedures. All foods were obtained from local markets. In general, foods were cooked on

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standard electric stoves. The initial survey included two sets of cooking times and temperatures for each food. One set approximated common practice in the United States, and the other utilized more severe conditions that generally resulted in a very well done, though not burned, sample. In most cases, when significant mutagenic activity was detected in the initial screening, a more detailed investigation of the dependence of mutagenicity on cooking time and temperature was carried out. Cooking temperatures were monitored by calibrated copper-constantan thermocouples and recorded on a multipoint recorder (Speedomax 252, Leeds and Northrup, North Wales, PA). Pan temperatures normally dropped 40–100°C upon addition of the food. Except when mixtures of food were examined, no seasonings or other additives were used. When normal procedures required cooking oil, corn oil was used. For the initial surveys most samples were cooked in stainless-steel pans. The term broiling is used to describe direct exposure to a high-temperature heat source from above. Broiled samples were prepared in ceramic cooking dishes. The more detailed time and temperature investigations of frying conditions were conducted on an electrically heated stainless-steel griddle (Model EL 124, Cecilware Corp., Long Island City, NY).

Beef. Ground round-steak patties (100 g, 11 cm diameter × 1 cm thick, c. 15% fat) were fried; choice round steaks were pan-fried, oven-broiled, baked, stewed and braised under the conditions given in Fig. 1. Patties were fried on the stainless-steel griddle. Steaks were fried on a stainless-steel surface by first searing the meat for a short time at high heat in a covered pan, followed by frying it on a griddle for an additional period at a lower temperature. Fats and cooking juices were removed during cooking.

Broiling of steaks took place in an aluminium-foil-lined pan placed 8 cm from the electric heating element. The steaks were turned and cooking juices were removed at 3-min intervals. For baking (roasting), meat samples were loosely rolled, tied with string, and cooked in foil-lined pans. Samples were turned every 15 min and juices were removed during cooking.

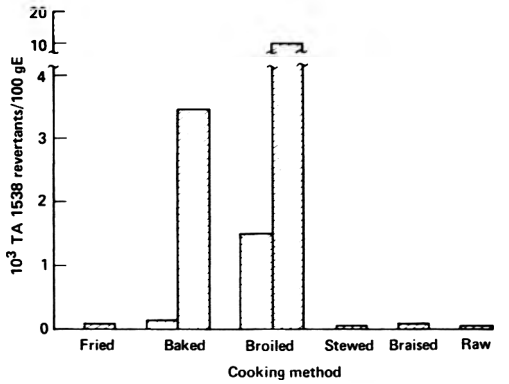

Fig. 1. Mutagen formation in beefsteak during different cooking processes: fried for 2·5 min at 190°C, followed by 7 min at 107°C on each side; baked for 60 min at 176°C (left) or 90 min at 176°C (right); broiled for 9 min/side at 300°C (left) or 11 min/side at 300°C (right); stewed for 165 min at 100°C; braised for 165 min at 100°C; raw, uncooked. gE = Gram equivalents of fresh uncooked food.

Stewed samples were prepared by slowly boiling diced meat (2- to 3-cm cubes) in water, with addition of water during cooking to maintain volume. Braised samples were prepared by first browning meat cubes (2–3 cm) in a frying pan and then slowly cooking the cubes in water in covered pots in an oven. Water level was maintained during cooking.

Pork products. Precooked and uncooked hams were sliced *c.* 1 cm thick and fried or broiled. Samples were turned once during cooking. Bacon slices (c. 3 mm thick) were fried in open pans and turned once during cooking. Loin centre cut pork chops (5–10 mm thick) were fried in an open pan and turned twice during cooking. Table 1 gives the time and temperature conditions for cooking these meats.

Temperature effects. A more detailed examination of the effect of temperature on mutagen production was conducted with ground beef, round steak, beef sausage, ham, pork chops, and bacon. Fig. 2 gives the time and temperature conditions of preparation.

Chicken. Figure 3 gives the conditions under which chicken samples were baked, pan-fried, deep-fried, and broiled. Dark meat was separated from light meat. Baked samples were prepared in uncovered pans and meat was turned over after approximately 20 min. Pan-fried samples were prepared by heating chicken in oil in uncovered pans. Deep-fried samples were prepared by immersion of the meat in cooking oil. Broiled chicken was prepared in open pans and the meat was turned once, approximately midway in the cooking process.

Eggs and egg products. Grade AA chicken eggs were cooked under household conditions and were included in mixtures in meringue, custard and pancakes. Eggs were fried on either one side or both sides on oiled frying pans. For baking, homogenized eggs were cooked in oiled Pyrex baking dishes. Meringue was prepared from egg whites, sucrose and cream of tartar by usual methods. Baked custard was prepared from whole eggs, sucrose and cows' milk by conventional procedures. Pancakes were prepared from batter containing sodium chloride, sucrose, baking powder, whole eggs, margarine and cows' milk. A more detailed examination was also conducted on the effect of cooking conditions on mutagen production in patties of whole egg, egg white and egg yolk. Figures 4 and 5 give the frying times and griddle temperatures. Egg patties were prepared by heating 60 ml of egg mixture or egg fraction for 1 hr at 95 C in an 8-cm-diameter petri plate, a procedure that by itself causes no mutagen to form.

Hamburgers. Samples of four hamburgers cooked in 'fast-food' restaurants near Berkeley, CA were obtained from each of seven different restaurants on three occasions over a 9-wk period.

Extraction procedures. Cooked samples were deboned if necessary, and when an obvious cooked outer portion or crust was present on the food, this material was separated from the inner portion (which was uniformly not mutagenic under our cooking conditions) and extracted for bioassay. Since the specific gram equivalents (gE) are always based on the initial uncooked wet weight the proportional weights of the outer and inner portions do not enter into the specific mutagenicity calculation. The organic solvent-soluble basic fraction from each food material was prepared

Table 1. *Mutagen formation in pork products under different cooking conditions*

Food	Cooking conditions: Method	Temperature (°C)	Time (min/side)	No. of TA1538 revertants/100 gE*
Ham	Griddle	200†	6	6000
Precooked ham	Pan fried	275‡	1·5	110
Precooked ham	Pan fried	275	3·5	4650
Precooked ham	Pan fried	275	10	16,700
Uncooked ham	Pan fried	275	3	630
Uncooked ham	Pan fried	275	5	850
Uncooked ham	Pan fried	275	10	24,010
Bacon	Pan fried	225	7	720
Bacon	Pan fried	225	10	1420
Pork chops	Pan fried	275	3	250
Pork chops	Pan fried	275	6	5500
Precooked ham	Broiled	300§ (125)‖	1	160
Precooked ham	Broiled	300 (125)	3	1850
Precooked ham	Broiled	270 (110)	7	16,500
Uncooked ham	Broiled	280 (110)	3	60
Uncooked ham	Broiled	280 (110)	6	1420
Uncooked ham	Broiled	280 (110)	9	6650

*Gram equivalents of fresh (uncooked) food.
†Griddle was thermostatically controlled; meat surface temperature during cooking was 20–40°C below griddle surface temperature.
‡Frying pan temperatures at the stove setting used for cooking; meat surface temperatures during cooking were 40–80°C lower.
§Broiler pan temperatures measured with meat removed.
‖Temperatures on meat surface during broiling.

after homogenizing the food and extracting with acetone. The extraction procedure is the same as previously used to extract mutagenic material from fried ground beef (Felton, Carrano, Carver *et al.* 1980).

Assay for mutagenic activity. After dissolving the basic fraction in DMSO, the mutagenic activity of the basic fraction was assayed in *Salmonella typhimurium* strain TA1538. Details of the procedure have been published (Felton *et al.* 1981). In brief, the basic fraction from each food dissolved in DMSO was combined with a culture of *S. typhimurium* strain TA1538 and metabolically activated by Aroclor-induced rat liver S-9 mix (2 mg protein/plate). Following the standard plating technique of Ames, McCann & Yamasaki (1975) the his$^+$ revertants were counted on a calibrated Biotran II (New Brunswick Sci. Co., Edison, NJ) automated colony counter. In all cases, the number of revertants induced by a dose of basic fraction equivalent to 100 g original fresh weight of food (100 gE) was extrapolated from the linear portion of dose response curves derived from duplicate platings of at least five doses. The coefficient of variation (standard deviation divided by the mean × 100) was 6% for five independent assays of a single sample of ground beef base fraction.

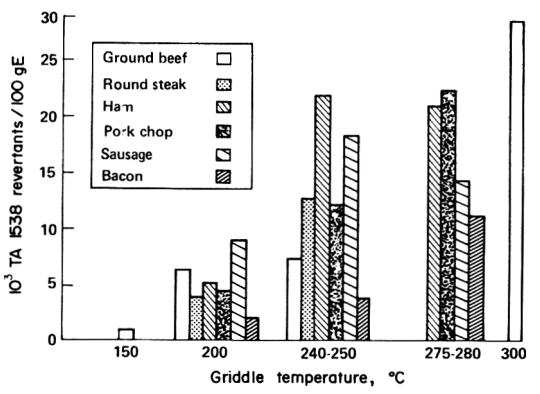

Fig. 2. Comparative mutagenesis of fried ground beef, round steak, ham, pork chop, sausage and bacon. All meats were fried for 6 min/side on a stainless-steel griddle. gE = Gram equivalents of fresh uncooked food.

RESULTS

An indication of the reproducibility of the cooking, extraction, and bioassay methods is given by the coef-

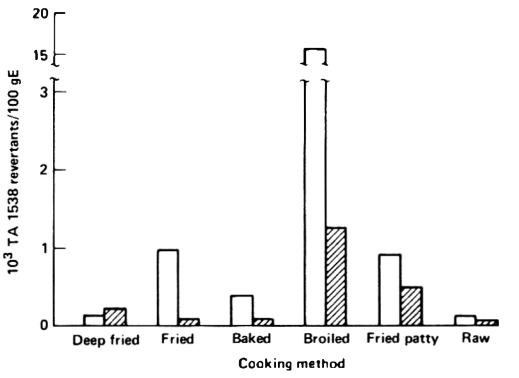

Fig. 3. Mutagen formation in chicken cooked by different methods. White (□) and dark (▨) meats were deep fried for 12 min at 101°C, fried for 15 min on one side and 10 min on the second side at 103°C, baked for 50 min at 190°C, broiled for 17 min/side at 274°C, fried in the form of a patty for 6 min/side at 200°C or left raw. gE = Gram equivalents of fresh uncooked food.

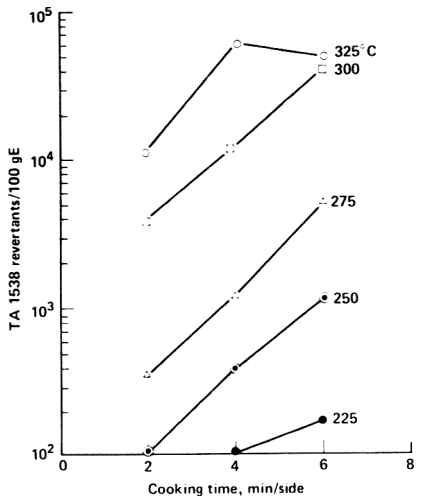

Fig. 4. Mutagen formation in egg patties fried at various temperatures as a function of time. A stainless-steel griddle was used to fry the eggs at 225, 250, 275, 300 or 325 C. gE = Gram equivalents of fresh uncooked food.

ficient of variation of the number of revertants per 100 g equivalents (gE) of fresh food following replicate platings. For six independent preparations of ground beef fried at 200°C for 6 min per side, the coefficient of variation was 19%. For all foods tested no significant mutagenesis was seen when the food was uncooked.

For round steak (Fig. 1) extensive baking and broiling caused extensive mutagen formation (up to 10,000 revertants/100 gE). Note that the conditions for frying round steak were milder than those generally used for ground beef (Felton *et al.* 1981). Slow cooking of beefsteak at lower temperatures, by stewing and braising, yielded fewer than 200 revertants/100 gE.

Figure 2 compares the mutagenic activity of red meats when fried for 6 min per side. In general, mutagenic activity increases with temperature. Ham and sausage reach a maximum yield at 240–250°C, whereas pork chop and bacon continue to increase in yield through 275–280°C. Bacon exhibits less activity than the other meats, but it also has the lowest protein content. Table 1 presents additional data on pork products.

Chicken (Fig. 3) showed extensive mutagen formation after both white and dark meat were broiled. White meat that was fried also showed a moderate level of activity: 1000 revertants/100 gE. Baked and deep fat-fried chicken showed much less activity, although significantly above background. Dark chicken meat contained less activity than white meat except following deep-fat frying, when both were only slightly mutagenic. To test whether this difference could be due to the different shapes of pieces of white and dark chicken meat used in frying, samples of each were ground and formed into patties. After frying at 200°C for 6 min per side, the white meat induced 810 revertants/100 gE and the dark meat induced 350 revertants/100 gE.

Table 2 shows that very little mutagen is formed in eggs and egg products unless severe cooking conditions are used. The positive samples, eggs fried sunny-side up at 310°C for 6 min and pancakes cooked at a high temperature, did not approach the values found in meats cooked at lower temperatures for shorter periods. Baked meringues and custards and pancakes fried at 350°C were non-mutagenic.

Certain foods, ham, sausage, bacon and pork chops were tested for mutagenicity with the base-substitution sensitive strain TA100. It was reasoned that if these foods contained different mutagenic components from ground beef [ground beef gave barely double the background revertant frequency with TA100 and only in the preincubation assay (Felton *et al.* 1981)], then they might possibly be mutagenic in TA100 but this turned out not to be the case. Sausage cooked at 280 C for 8 min did not significantly increase the number of revertants above the background level with TA100. Ham cooked at 280°C for 6 min, and bacon and pork chops cooked at 280°C for 8 min increased the number of revertants to 2·5, 2 and 6 times the background level, respectively. In strain TA1538, pork chops cooked under the same conditions yielded revertants at 2000 times the background level.

One increasingly prevalent source of protein intake in the United States is the grilled hamburger cooked in 'fast-food' restaurants. Of the seven restaurants we sampled, two provided hamburgers with consistently high mutagen content, three with consistently low mutagen content, and two showed considerable variation between sampling times (Table 3). Precise data on the cooking times and temperatures of the hamburgers are not available. However, discussion with each vendor indicated a qualitative correlation of

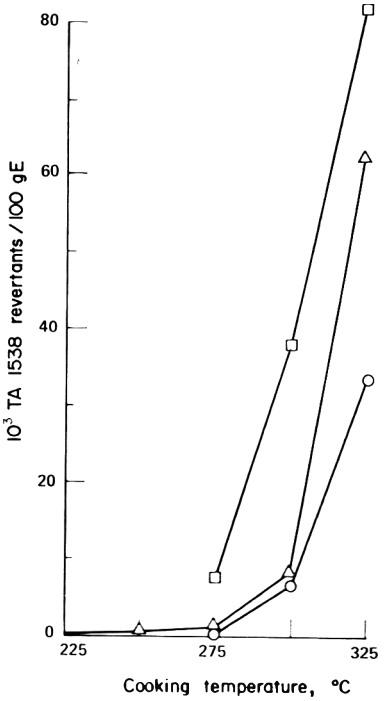

Fig. 5. Mutagen formation in fried egg patties as a function of temperature. Patties of whole egg (△), yolk (□) and white (○) were fried for 4 min side on a stainless-steel griddle. gE = Gram equivalents of fresh uncooked food.

Table 2. *Mutagen formation in eggs and egg products*

Sample	Cooking conditions: Method	Temperature (°C)	Time (min)	No. of TA1538 revertants/100 gE*
Eggs	Fried—unturned	177	4	NS
		310	6	290
Eggs	Fried—turned	210	2·7	NS
		310	6	NS
Eggs	Hard-boiled	100	15	NS
		100	35	NS
		100	50	NS
Eggs	Baked/shirred	163	25	NS
		190	60	NS
Meringue	Baked	135	90	NS
		177	90	NS
Custard	Baked	177	75	NS
		204	75	NS
Pancakes	Fried	350	4	NS
		475	3	190

*Gram equivalents of fresh (uncooked) food.

NS = Not significantly different from the spontaneous reversion rate, based on failure to meet two separate criteria: first, that the highest dose point be greater than two standard deviations above the historical spontaneous rate for our laboratory (22 ± 6·2 SD) and second, that the linear regression fit to the data show a significant slope at the 95% confidence level for the doses included.

higher mutagen content with more severe cooking conditions, including the use of increased temperature associated with the sporadic high values occurring when the cook was rushed. As a semi-quantitative measure of the extent of cooking, the residual water content of the samples was determined. Although there seemed to be a relationship, a chi-square test relating residual water to mutagen content did not show significance at the 5% level.

Table 3. *Mutagenicity of the basic fraction from commercially cooked hamburgers*

Vendor	Visit	No. of TA1538 revertants/100 g cooked weight
A	1	2300
	2	1100
	3	5900
B	1	280
	2	2630
	3	150
C	1	120
	2	320
D	1	NS
	2	NS
	3	180
E	1	2290
	2	4640
	3	4070
F	1	510
	2	270
	3	370
G	1	810
	2	5000
	3	310

NS = Not significantly above spontaneous reversion frequency (see footnote in Table 2).

DISCUSSION

This survey of the major sources of cooked protein foods in the American diet shows that most meats, when cooked to a well-done but noncharred state, contain mutagens active in the Salmonella bioassay. Eggs cooked under severe conditions also contain mutagen activity. Although mutagen formation in the foods investigated here may be sharply reduced or eliminated by minimizing the severity of the cooking conditions, such minimal conditions are not likely to be used in most household cooking procedures. For example, ground beef and egg patties fried for reasonable periods (< 10 min/side) at pan temperatures less than 150°C and 250°C, respectively, are only weakly mutagenic. However, few commonly used cooking utensils give accurate control of pan temperature, and thus, the heat settings normally used to obtain intermediate frying temperatures on electric ranges (low–medium) can result in pan temperatures that are well into the mutagen-producing range (200–300°C). Furthermore, pan temperatures on gas ranges are likely to reach even higher temperatures than those encountered on electric stoves because the gas burners are generally not thermostatically controlled. Thus, if the results of future studies show that human exposure to mutagens from cooked protein-rich foods must be reduced, substantial modifications in cooking techniques and/or diet may be required.

The first signs of charring on samples of chicken, eggs, beef and pork were associated with high levels of activity. For most of these samples, mutagenicity tended to increase with increased charring. Exceptions to this trend were the mutagen contents of the ground meats, which tended to decrease after reaching a maximum, although the degree of charring continued to increase with increased cooking time. This

difference in behaviour of ground and unground meat might be due to the formation of an obvious, distinct crust on the ground meats, which appears to inhibit further heat transfer to the interior of the meat. Thus, prolonged heating of ground meat might destroy or volatilize (Rappaport, McCartney & Wei, 1979) mutagens produced within a relatively restricted outer layer of the patties. Since unground meats do not form such distinct crusts, mutagen formation might occur at increasing depths in the meat as the cooking time increases.

It is interesting to compare fried egg and beef at both 200 and 300°C. Significant mutagen formation occurs at lower temperatures in ground beef (Fig. 2), yielding 6300 revertants/100 gE at 200°C, compared to no detectable increase over background for eggs at 200°C. But at 300°C whole egg has accumulated 40,000 revertants/100 gE when cooked 6 min/side compared to 30,000 revertants/100 gE for beef. Egg yolk is more active than egg white, with 40,000 revertants/100 gE generated in 4 min. This comparison suggests two important points. First, foods found to lack mutagenicity at 200°C may contain substantial amounts of mutagen if cooked at higher temperatures. Second, foods giving high revertant rates at low temperatures do not necessarily show proportionately higher levels when cooked at higher temperatures.

According to the USDA and HEW (HANES) surveys 58% of the average daily protein intake of an American is included in nine major food items: ground beef, beef steak, eggs, pork chops, fried chicken, pot-roasted or braised beef, ham, roast beef and bacon (Plumlee *et al.* 1982). The remaining protein is derived from milk, some of which may contain mutagen (Green, Ben-Hur, Riklis *et al.* 1980), bread, and peanut butter (although the latter two foods may be baked, toasted or roasted, they are not cooked in the same manner or to the degree used for meat and eggs), and from minor sources including fish (see accompanying paper).

The total amount of mutagenic activity (assayed with *S. typhimurium* TA1538) that is ingested from cooked proteins in an average well-cooked American diet is equivalent to approximately 5600 revertants/day. Calculations were made from data presented in this paper for normal cooking to a well-done, non-charred state. The major contributions come from ground beef, beefsteak, pork chops, ham and roast beef. Eggs, fried chicken and bacon contribute much less mutagenic activity and beef pot roast makes a negligible contribution. It is difficult to estimate the overall exposure of the American population, owing to an unfortunate lack of available data on cooking temperatures used in the household and on the proportion of people that cook their foods well-done or overdone.

This survey of the effects of cooking the major protein foods of the diet will serve as a guide to the relative tendencies of particular foods and cooking conditions to induce mutagen formation. However, it is only a preliminary step in determining whether a significant health hazard results from cooking. Much more information must be gathered before any assessment of human risk from mutagens produced by cooking foods will be feasible. It is necessary to isolate and identify the mutagens (several are present) and to determine their potencies in various kinds of short-term mammalian bioassays. Furthermore, it is important to understand the behaviour of the mutagens within the gastro-intestinal tract (their stability and transformations, the effects of anti- and co-mutagens and the extent of absorption into the intestinal mucosa, and transport to other organs), as well as whether metabolic activation to ultimate mutagenic forms occurs *in vivo* in mammals. Finally, if progress along these lines continues to suggest a potential hazard, then the mutagens will presumably have to undergo bioassays for carcinogenesis and heritable genetic damage in preparation for a formal risk assessment. At the present time bacterial mutagenesis data suggest that continued research is important. However, any extrapolation regarding the existence of a human health hazard is premature and unwarranted.

Acknowledgements—The authors wish to thank Dr M. L. Mendelsohn for helpful discussion, and C. Hein, L. Quong, B. Yee, K. Aikawa, M. Ma, M. Madrigal, J. Lew, K. Forsman and K. Cavanaugh for their valuable technical assistance. This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48 and supported by an Interagency Agreement (NIEHS 222Y01-ES-80038) between the National Institute of Environmental Health Sciences and the Department of Energy.

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0278-6915/82/040365-05$03.00/0

MUTAGENS FROM THE COOKING OF FOOD. III. SURVEY BY AMES/SALMONELLA TEST OF MUTAGEN FORMATION IN SECONDARY SOURCES OF COOKED DIETARY PROTEIN

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(*Received 25 August 1981*)

Abstract—A survey of mutagen formation during the cooking of a variety of protein-rich foods that are minor sources of protein intake in the American diet is reported (see Bjeldanes, Morris, Felton *et al.* (1982) for survey of major protein foods). Milk, cheese, tofu and organ meats showed negligible mutagen formation except following high-temperature cooking for long periods of time. Even under the most extreme conditions, tofu, cheese and milk exhibited fewer than 500 Ames/*Salmonella typhimurium* revertants/100 g equivalents (wet weight of uncooked food), and organ meats only double that amount. Beans showed low mutagen formation after boiling and boiling followed by frying (with and without oil). Only boiling of beans followed by baking for 1 hr gave appreciable mutagenicity (3650 revertants/100 g equivalents). Seafood samples gave a variety of results: red snapper, salmon, trout, halibut and rock cod all gave more than 1000 revertants/100 g wet weight equivalents when pan-fried or griddle-fried for about 6 min/side. Baked or poached rock cod and deep-fried shrimp showed no significant mutagen formation. Broiled lamb chops showed mutagen formation similar to that in red meats tested in the preceding paper: 16,000 revertants/100 g equivalents. These findings show that as measured by bioassay in *S. typhimurium*, most of the foods that are minor sources of protein in the American diet are also minor sources of cooking-induced mutagens.

INTRODUCTION

This paper is a continuation of our survey of mutagen formation during the cooking of the principal sources of protein in the North American diet (Bjeldanes, Morris, Felton *et al.* 1982). Here we present the results from a variety of protein-rich foods that are commonly consumed, but do not appear in the list of major sources of cooked protein foods (Plumlee, Bjeldanes & Hatch, 1981).

EXPERIMENTAL

Cooking procedures. All foods were obtained from local markets. In general, foods were cooked on standard electric ranges. Two sets of cooking times and/or temperatures were generally used for each food. One set of conditions was considered normal or common for the United States, and the other set was considered severe and generally resulted in a very well-done, but not burned, sample. Cooking temperatures were monitored by calibrated copper-constantan thermocouples and recorded on a multipoint recorder (Speedomax Model 252, Leeds and Northrup, North Wales, PA). The temperatures given in the tables were measured in each method with the empty utensil, on top of a household electric range, at the heat control setting that was used in cooking the food sample. The single exception was griddle-fried rock cod, which was cooked under thermostatic feedback control on the experimental electric griddle used for time-temperature studies.

The actual cooking temperatures for the items cooked with the household ranges were much lower than those recorded for the empty utensils, owing to the cooling effects of the food when added and to the evaporation of water. In general, the initial cooking temperatures were 40–100 C lower than pan temperatures, with a progressive rise during cooking. Except when food mixtures were examined, no seasonings or other additives were used. When normal procedures required cooking oil, corn oil was used. Most stove-top cooking was done in stainless steel pans. The term broiling is used to describe direct exposure to a high-temperature heat source from above. Broiled samples were prepared in ceramic cooking dishes.

Milk. Samples of approximately 40 g of whole cows' milk were refluxed vigorously for 5, 15, 30, 60 and 240 min. Similar-size samples were reduced to 50% and 25% of the original volume by vigorous boiling.

Cheese. Samples, 3–4 mm thick, of sharp cheddar and American pasteurized process cheeses were fried or baked in an electric oven under the conditions indicated in Table 1.

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Table 1. *Mutagen formation in cheese samples under different cooking conditions*

Sample no.	Type of cheese	Cooking conditions			No. of TA1538 revertants/ 100 gE*
		Method	Time (min)	Temperature (°C)	
C16	Sharp cheddar	Baked	15	204	NS
C17	Sharp cheddar	Baked	30	232	63
C18	Sharp cheddar	Fried	5	240	NS
C19	Sharp cheddar	Fried	5	375	NS
C20	American	Baked	10	204	NS
C21	American	Baked	20	232	360
C22	American	Fried	5	240	63
C23	American	Fried	5	375	94

NS = Not significantly different from the spontaneous reversion rate, based on failure to meet two separate criteria: that the highest dose point be more than two standard deviations above the historical spontaneous rate for our laboratory (22 ± 6·2) and that the linear regression fit to the data shows a significant slope at the 95% confidence level for the doses included.
*Gram equivalents of fresh (uncooked) food.

Beans. Samples of kidney and pinto beans were cooked by these methods: boiling only, boiling followed by frying with and without oil, and boiling followed by baking, under the conditions indicated in Table 2. Bean samples (230 g) were soaked overnight in tap water (500 ml) in an aluminium pot at 10°C. Soaked beans were then boiled with added water (500 ml). Water level was maintained during the cooking period by adding tap water. Fried beans were prepared by heating boiled mashed beans in an open frying pan, either with or without a small amount of cooking oil. Baked beans were prepared by cooking whole boiled beans in a baking dish covered with aluminium foil.

Tofu. Although tofu is not a major protein source in the American diet, it is used heavily in certain segments of the population and was therefore subjected to mutagen assay. Under the conditions indicated in Table 3, regular tofu samples were deep-fried, steamed, stir-fried, baked, or simmered, and soft tofu samples were boiled. Deep-fried, stir-fried, water-simmered, and boiled samples were prepared by common procedures from diced (5 × 5 × 3 cm) and drained tofu. Steamed and baked samples were prepared with mashed tofu. For baking, samples were formed into loaves or patties and cooked on oiled pans.

Lamb. Although lamb does not appear on the list of major protein sources, it is consumed in the USA. Lamb was prepared under the conditions shown in Table 4.

Organ meats. Beef liver, heart, kidney, tongue, and brain were prepared under the conditions indicated in Table 5. Liver samples were broiled, braised, or fried. Broiled samples were prepared from meat (1 cm thick)

Table 2. *Mutagen formation in bean samples under different cooking conditions*

Type of bean	Cooking conditions			No. of TA1538 revertants/ 100 gE*
	Method	Time (min)	Temperature (°C)	
Pinto	Boiled	39	100	NS
Pinto	Boiled	78	100	75
Kidney	Boiled	45	100	190
Kidney	Boiled	90	100	140
Pinto	Boiled/fried†	2	450	NS
Pinto	Boiled/fried†	4	450	NS
Kidney	Boiled/fried†	5	450	NS
Kidney	Boiled/fried†	10	450	380
Pinto	Boiled/baked‡	60	176	3650
Pinto	Boiled/baked‡	120	176	460
Kidney	Boiled/baked‡	60	176	140
Kidney	Boiled/baked‡	120	176	140
Pinto	Boiled/fried§	4	450	NS
Pinto	Boiled/fried§	8	450	NS
Kidney	Boiled/fried§	4	450	58
Kidney	Boiled/fried§	8	450	150

NS = Not significantly different from the spontaneous reversion rate (See Table 1).
*Gram equivalents of fresh (uncooked) food.
†Boiled for 60 min and then fried for the time given without oil.
‡Boiled for 60 min and then baked for the time given.
§Boiled for 60 min and then fried with oil.

Table 3. *Mutagen formation in tofu samples under different cooking conditions*

Type of tofu	Cooking conditions: Method	Time (min)	Temperature (°C)	No. of TA1538 revertants/ 100 gE*
Regular	Deep-fried golden	10–12	162	NS
Regular	Deep-fried golden	10–12	162	NS
Regular	Deep-fried brown	15	162	400
Regular	Deep-fried brown	15	162	NS
Regular	Steamed	12	100	NS
Regular	Steamed	40	100	NS
Regular	Steamed	60	100	NS
Regular	Stir-fried	2	200	NS
Regular	Stir-fried	6	200	NS
Regular	Baked, loaf	60	176	NS
Regular	Baked, patties	20–25	176	NS
Sun-Tong Lee (soft)	Boiled	30	100	NS
Soft	Boiled	5	100	NS
Regular	Simmered	20	100	NS
Regular	Uncooked	0	—	NS

NS = Not significantly different from the spontaneous reversion rate (see Table 1).
*Gram equivalents of fresh (uncooked) food.

brushed with cooking oil. Braised and fried samples were prepared with slices (1·5 to 2·0 cm thick) coated with flour. Heart samples were baked whole on a rack or braised as previously described for beef (Bjeldanes *et al.* 1982). Kidneys were either cut in half and baked in an uncovered pan or sliced (1 cm thick) and sautéed in oil in a frying pan. Beef tongue was first simmered, cleaned of small bones and gristle, boiled, and then simmered again in an uncovered pan. Beef brain was soaked first in mild aqueous acid solution, then skinned and soaked in warm water, and finally blanched in mild acid solution. The product was either dried, rolled in flour and fried in a covered pan, or brushed with oil and broiled.

Seafood. Samples of rock cod fillets were baked, pan-fried, poached or broiled. Steaks or fillets of halibut, trout, salmon, and red snapper were pan-fried. Shrimp samples were deep-fat fried. The cooking conditions are indicated in Table 6. Baked samples were prepared in uncovered pans and the fish was not turned over during cooking. Fried samples were cooked in oil 3 mm deep. Poached fish was prepared by cooking samples in boiling water in an open pan and samples were not turned over. Broiled samples were prepared in greased, uncovered pans. Deep-fried shrimp was prepared by immersing shrimp in cooking oil. Batter-fried samples were obtained on different days from a local seafood galley.

Table 4. *Mutagen formation in broiled lamb chops under different cooking conditions*

Cooking conditions: Method	Time (min/side)	Temperature (°C)	No. of TA1538 revertants/ 100 gE*
Pan-broiled	5	210	14,100
Pan-broiled	6·5	210	16,700
Oven-broiled	4	225	390
Oven-broiled	10	280	680

*Gram equivalents of fresh (uncooked) food.

Extraction and mutagen assay procedures. When an obviously browned outer portion or crust was present on the cooked food, this material was separated from the inner portion and extracted for bioassay. Bioassay of the inner portions of foods were uniformly negative except for small amounts of mutagenic activity that appeared at extremely hot temperatures.

Foods were homogenized and extracted with acetone as previously described (Felton, Healy, Stuermer *et al.* 1981). After solvent concentration, samples were sequentially extracted with organic solvent at acidic and alkaline pH. The organic base fraction, which contained all of the detectable mutagens, was dissolved in DMSO and assayed with *Salmonella typhimurium* tester strains TA1538 or TA98. Aroclor-induced rat liver S-9 mix was used at a protein concentration of 2 mg/plate. The number of revertants induced/100 g wet weight equivalents (gE), of uncooked food, was extrapolated from the linear portions of dose-response curves as described in the preceding paper (Bjeldanes, Morris, Felton *et al.* 1982).

RESULTS

Tables 1–6 present the results of initial screening of cooked foods for mutagenic activity with *S. typhimurium* tester strains TA1538 or TA98. Whereas all of the foods tested developed mutagenic activity under some cooking conditions, only the most severe conditions produced activity in milk, cheese, beans, tofu, and organ meats. None of the uncooked foods showed any significant mutagenicity. The only mutagenic milk sample, the one boiled down to 25% of its original volume (440 revertants/100 gE) was thick and yellow, and burned material adhered to the cooking container. The rest of the milk samples were not discoloured and did not contain any mutagens. The most mutagenic cheese samples (Table 1, C21 and C23) had dark brown to black crusts and were considered overdone. The

Table 5. *Mutagen formation in beef organ meats under different cooking conditions*

Organ	Cooking conditions: Method	Time (min)	Temperature (°C)	No. of TA1538 revertants/ 100 gE*
Liver	Broiled	3/side	260	NS
Liver	Broiled	5/side	260	NS
Liver	Braised	2·5/20†	450/250†	210
Liver	Braised	2·5/30†	450/250†	79
Liver	Pan-fried	3/side	310	NS
Liver	Pan-fried	4·5/side	310	210
Heart	Baked	120	163	NS
Heart	Baked	165	163	81
Heart	Braised	2·5/90†	450/275†	NS
Heart	Braised	2·5/135†	450/275†	230
Kidney	Baked	60	149	310
Kidney	Baked	90	149	NS
Kidney	Sautéed	5	450	160
Kidney	Sautéed	7	450	830
Tongue	Boiled	120	100	NS
Tongue	Boiled	180	100	NS
Brains	Sautéed	2/10†	450/100†	260
Brains	Sautéed	2/15†	450/100†	920
Brains	Broiled	8/side	260	NS
Brains	Broiled	12/side	260	85

NS = Not significantly different from the spontaneous reversion rate (see Table 1).
*Gram equivalents of fresh (uncooked) food.
†In these cases, initial cooking at the first temperature shown was followed by further cooking at the second temperature shown.

Table 6. *Mutagen formation in seafood samples under different cooking conditions*

Type of fish	Cooking conditions: Method	Time (min)	Temperature (°C)	No. of TA1538 revertants/ 100 gE*
Rock cod	Pan-fried	4/side	280	NS
Rock cod	Pan-fried	8/side	280	180
Rock cod	Griddle-fried	6/side	200	1330
Rock cod	Baked	30	176	NS
Rock cod	Baked	30	204	NS
Rock cod	Baked	45	176	NS
Rock cod	Baked	45	204	NS
Rock cod	Poached	5	100	NS
Rock cod	Broiled	6/5†	300	NS
Rock cod	Broiled	12/10‡	300	1990
Sole	Pan-fried	1/side	280	120
Sole	Pan-fried	2/side	280	170
Halibut	Pan-fried	3·5/side	280	390
Halibut	Pan-fried	5·5/side	280	1080
Trout	Pan-fried	4/side	280	990
Trout	Pan-fried	6·5/side	280	3100
Salmon	Pan-fried	2·5/side	280	320
Salmon	Pan-fried	6/side	280	2800
Red snapper	Pan-fried	2·5/side	280	930
Red snapper	Pan-fried	5·5/side	280	2500
Shrimp	Deep-fat fried	9	190	NS
Shrimp	Deep-fat fried	15	190	NS
Seafood galley	Batter-fried§			180

NS = Not significantly different from the spontaneous reversion rate (see Table 1).
*Gram equivalents of fresh (uncooked) food.
†Six minutes on the first side and five minutes on second side.
‡Twelve minutes on the first side and ten minutes on second side.
§Commercially cooked.

nonmutagenic cheese samples did not have a burned appearance. The mutagenic tofu sample (Table 3) was dark brown, whereas the nonmutagenic samples were not markedly discoloured. The griddle-fried and the oven-broiled 10–12 min/side samples of rock cod showed moderate formation of mutagen (1330–1990 revertants/100 gE; Table 6). The more extensively fried trout, salmon, and red snapper yielded 2500-3100 revertants/100 gE, while halibut yielded 1080 revertants/100 gE. The remainder of the rock cod samples, and the sole, shrimp, and batter-fried seafood from the commercial galley exhibited negligible or small amounts of mutagen.

Preparation of beans and beef organ meats under several relatively moderate conditions produced low or moderate levels of mutagenic activity. None of the bean samples were burned or considered inedible. Several samples were weakly mutagenic (Table 2) and one sample contained considerable mutagenic activity. Many of the organ meat samples showed weak activity (Table 5), sautéed kidney and brain samples being the most active. None of the organ meat preparations were charred. The basic fractions from organ meats, rock cod, cheese and milk were tested with *S. typhimurium* strain TA100 following the most extreme cooking conditions. No significant mutagenesis was seen (data not shown).

DISCUSSION

The results show that some classes of cooked foods exhibited more mutagenic activity than other foods cooked to a similar degree. The most severely treated samples of milk, cheese, tofu, and rock cod, although slightly charred and hardly edible, were only slightly to moderately mutagenic. Less severely treated samples were not active.

There is no obvious explanation for the relatively low mutagen content of severely treated rock cod samples. Several of these samples were brown; many were overcooked. Thus, the browning reaction (Reynolds, 1965) in protein-rich carbohydrate-poor food need not necessarily yield mutagens. Since the amino acid composition of edible fish is similar to that of beef muscle (Food Policy and Food Sciences Service, Nutrition Division, Food and Agriculture Organization of the United Nations, 1970), substances other than or in addition to amino acids may be involved in mutagen formation in meats, at least in a soluble system after boiling of meat (Taylor, Fultz & Shore, 1981). There was an indication that fish containing more pink or red colour were more susceptible to mutagen formation than the white fish. This is in contrast to chicken, in which white meat forms more mutagen than dark meat cooked under the same conditions (see Bjeldanes *et al.* 1982).

The results indicate that the extent of mutagen formation in cooked food is related to cooking method as well as to the type of food. In general, cooking methods that use temperatures near 100°C result in low or nondetectable activity. The processes of stewing, boiling, and poaching, which cook near 100°C, result in low mutagenic activity regardless of the type of food. It has been reported that microwave cooking, also a low-temperature process, produces no mutagenic activity (Dolara, Commoner, Vithayathil *et al.* 1979).

Green, Ben-Hur, Riklis *et al.* (1980) have shown that milk sterilization conditions used in Israel—a brief period at ultra-high temperature followed by 20 min at 134°C—may produce a low level of mutagenic activity in commercial milk. American milk is generally pasteurized at 71°C for 30 min; we did not observe any significant mutagenic activity either in fresh milk or after boiling milk for as long as 4 hr.

Cooking methods such as oven roasting and baking, which heat food by indirect convection, appeared to produce low to intermediate levels of mutagenic activity in most foods. Frying and broiling, cooking procedures that heat foods by conductive and radiative processes, tended to be associated with the highest levels of mutagenicity. Thus, the rates of mutagen production in the tested foods become appreciable at temperatures greater than 100°C, and are greatly increased by cooking methods that use direct high-temperature processes.

Acknowledgements—The authors wish to thank Dr M. L. Mendelsohn for helpful discussion, and C. Hein, L. Quong, B. Yee, K. Aikawa, M. Ma, M. Madrigal, J. Lew, K. Forsman, and K. Cavanaugh for their technical contribution. This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48 and supported by an Interagency Agreement (NIEHS 222Y01-ES-80038) between the National Institute of Environmental Health Sciences and the Department of Energy.

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Fd Chem. Toxic. Vol. 20, pp. 371 to 382, 1982
Printed in Great Britain.

0278-6915/82/040371-10$03.00/0

A COMPARISON OF THE EFFECTS OF LACTOSE AND OF TWO CHEMICALLY MODIFIED WAXY MAIZE STARCHES ON MINERAL METABOLISM IN THE RAT

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(*Received 15 June 1981; revision received 20 January 1982*)

Abstract—Diets containing 30% by weight of waxy maize starch, lactose monohydrate, acetylated distarch phosphate (EEC No. 1414) or acetylated distarch adipate (EEC No. 1422) were fed to weanling female Specified Pathogen-Free Sprague–Dawley rats for 1 yr and to similar 9-month-old rats for 34 wk. Behaviour and general health were unaffected by the different diets and there were no diet-related differences in food consumption. At the end of the experiment with 9-month-old rats the mean body weight of the animals receiving lactose was significantly lower than that of the controls receiving starch. The animals receiving the modified starches were slightly but not significantly heavier than the controls at the end of both experiments. The main treatment-related changes in rats on the three test diets were (1) caecal enlargement, (2) increased urinary excretion of calcium, (3) increased renal calcification as measured by chemical analysis of renal tissue obtained at autopsy and (4) increased medullary and pelvic nephrocalcinosis as assessed histopathologically. Acetylated distarch adipate had a slightly greater effect on the above parameters than acetylated distarch phosphate but both modified starches had less effect than lactose. The calcium content of the kidneys, as measured by chemical analysis or histopathology, increased with age, even in the animals receiving the control diet. This change may be due to excessively high concentrations of calcium and phosphorus in all the diets, including the control diet. Cortico-medullary mineral deposits were not a feature in these studies possibly because the diets were not deficient in magnesium. The importance of correct dietary formulation in long-term toxicity studies is emphasized.

INTRODUCTION

There have been several reports that chemically modified starches such as acetylated distarch adipate or hydroxypropyl distarch phosphate cause an enlargement of the caecum and the formation of calcareous deposits within the renal pelvis when introduced into the diet of rats in place of natural starch. Bailey, Cox & Morgareidge (1973), in a 90-day study with weanling Wistar rats, compared the effects of a diet containing up to 25% by weight of hydroxypropyl distarch phosphate with that of a normal diet containing 25% of unmodified starch. Enlarged caeca and calcareous deposits within the renal pelvis and/or pelvic epithelium were reported in 40% of the test animals but not in the control group. Similar findings were reported by de Groot, Til, Feron *et al.* (1974) in a 2-yr study on rats and by Feron, Til & Immel (1978) in an 89-wk study on mice. In addition to enlargement of the caecum and colon and an increased incidence of intratubular nephrocalcinosis, these studies revealed a slight growth retardation, increased urinary excretion of calcium, magnesium and phosphorus and a slightly increased incidence of concrements in the renal pelvic space or bladder. Truhaut, Coquet, Fouillet *et al.* (1979) on the other hand, found that in comparison with unmodified starch there were no significant differences in rate of body growth, serum biochemistry or organ weights in pathogen-free Sprague–Dawley rats given a diet containing 62% of acetylated distarch adipate or acetylated distarch glycerol for 2 yr. Hyperplasia of renal papillary and pelvic epithelia, accompanied by calcified patches in the underlying tissues, were observed but they occurred with approximately equal frequency in both the control and test groups. Some of these discrepancies may be due to differences in the type of starch used but the absence of data relating to organ weights or urine chemistry from the study of Truhaut *et al.* (1979) make it difficult to compare their results with those of previous workers.

It has long been recognized that high dietary intakes of carbohydrates and particularly lactose, increase the absorption of calcium (Bergeim, 1926; Greenwald & Gross, 1929). Other effects include caecal enlargement, increased urinary excretion of calcium, hyperplasia of renal papillary or pelvic epithelia and an increase in the frequency and severity of calcium deposits in the kidney (de Groot & Feron, 1975/6; Feron *et al.* 1978; Vaughan & Filer, 1960).

In the present study we have examined the possibility that chemically modified starches may resemble lactose in their effects on calcium metabolism.

Abbreviations: DS = Degree of substitution; E1414 = Acetylated distarch phosphate; E1422 = Acetylated distarch adipate.

Table 1. *Specifications of test and control starches and of lactose*

Parameter	Control starch	E1414	E1422	Lactose
Ca (ppm)	165	170	260	11
Mg (ppm)	115	70	90	3
P (ppm)	39	154	37	42
Acetyl (% w/w)	—	1·2%*†	2·1%‡	—
Degree of substitution§	—	0·05	0·08	—

*Analysis by Corn Products (Europe) Ltd, Avenue Louise 149, Bte 13, B-1050, Brussels, Belgium.
†Analysis by Scholten-AVEBE, 9607 PN, Foxhol, The Netherlands.
‡Analysis by Laing National (UK) Ltd, Trafford Park, Manchester M17 1BJ.
§The degree of substitution (DS) is the average number of substituents per D-glucose unit of the starch.

EXPERIMENTAL

Test materials. The two chemically modified starches studied were pregelatinized acetylated distarch phosphate (EEC No. 1414) and pregelatinized acetylated distarch adipate (EEC No. 1422). These and the pregelatinized unmodified waxy maize starch were supplied by the Association des Amidonnaires de Mais de la C.E.E., Brussels. Lactose monohydrate (USP XIX/EP 73; 99·8%) was supplied by De Melkindustrie Veghel, Holland. Further details of the specification of the three starches and of the lactose, are given in Table 1.

Animals. Female specified pathogen-free Sprague–Dawley rats, obtained from Charles River, Manston, Kent were used. For Experiment I the animals were aged 3–4 weeks when they were received from the supplier. For Experiment II the animals were ex-breeders, aged 9 months when they were received.

Diets. The diets were prepared in pelleted form, by the Central Institute for Nutrition and Food Research (CIVO), Zeist, Holland. The basic diet used by de Groot *et al.* (1974) formed the basis for all the diets used in the present studies. The control diet comprised 8% fish, 4% meat scraps, 20% soya-bean meal, 7% maize meal, 20% wheat meal, 3% grass meal, 3% brewer's yeast, 0·2% B-vitamins (in mg/kg diet: thiamine HCl, 4; riboflavin, 5; pyridoxine HCl, 2·5; niacin, 2·5; Ca-pantothenate, 15; biotin, 0·1; folic acid, 1·0; vitamin B_{12}, 0·025), 0·4% vitamin A, D_3, E preparation (in IU/g preparation: vitamin A, 2250; vitamin D_3, 750; vitamin E, 25), 0·5% steamed bone meal, 0·4% KH_2PO_4, 0·5% trace mineralized salt (percentage composition: MnO, 2; $ZnCl_2$, 0·05; KI, 0·012; Co acetate.$4H_2O$, 0·04; $FeSO_4.7H_2O$, 2·5; $CuSO_4.5H_2O$, 0·8; NaCl, 94·15) 3% margarine and 30% pregelatinized starch. Molasses was added (50 g/kg diet) as a binding agent for pelleting the diets. The diameter of the pellets was 10 mm. The test diets were prepared in the same way as the control diet and were identical with the control diet except that the 30% content of pregelatinized waxy maize starch was replaced by 30% pregelatinized acetylated di(waxy) starch phosphate (E1414), 30% pregelatinized acetylated distarch adipate (E1422) or 30% lactose and the minerals added to the test diets were adjusted where necessary by adding potassium hydrogen phosphate to raise the concentration of phosphorus, calcium carbonate to raise calcium concentration, and magnesium sulphate to raise magnesium concentration. Fresh batches of food were prepared every 4–8 wk and were stored at −20°C until used. The results of analysis of a typical batch are shown in Table 2.

Experimental design—Experiment I. One hundred weanling rats were allocated at random to four groups of 25 each: group 1, control; group 2, E1414; group 3, E1422; group 4, lactose. The animals were housed in wire-mesh cages in pairs or groups of three and were identified by cage number and tail markings made with coloured felt pens. All animals were weighed, initially at weekly intervals and later at fortnightly intervals. During an acclimatization period of a week all rats were fed on the control diet and were given free access to both food and distilled water.

Subsequently from what was designated day 1 of the experiment, thirteen animals in each group began to receive their respective diets and stayed in their normal cages throughout. One of these from each group was killed at weeks 5, 9, 12, 27, 31 and 39 to check for any progressive or age-related change. Additionally one control animal was killed at week 2 to check autopsy techniques. The remaining twelve animals in each group, divided into four groups of three, spent intermittent weekly stays in separate all-glass metabolism cages (Metabowl, Model 11, Jencons Ltd., Hemel Hempstead, England), which provided reliable measurement of food consumption and good separation of urine and faeces. The timing of these stays and of the switch to their respective diets is shown in Fig. 1. Thus the first three such rats in each group spent week 1 in metabolism cages, started their diet at the beginning of week 2, and subsequently spent weeks 5, 9, 23, 37 and 49 in metabolism cages, with successive groups of three rats following the same pattern but starting one week later. All surviving animals in all groups were killed at week 52.

Experimental design—Experiment II. The design followed essentially that of Experiment I, except that the rats used were approximately 9 months old at the start of the experiment, there were only two interim kills of one animal per group (at weeks 1 and 18) and the four subgroups of three animals from each group for metabolism studies were only observed over three 7-day periods covering weeks 1–4, 15–18 and 27–30. The same observations and measurements were made as in Experiment 1, surviving animals in all groups being killed at week 34. In both experiments, one group 2 metabolism animal died prematurely and a

Table 2. *Results of chemical analysis of blended diets*

Constituent	Control	E1414	E1422	Lactose
Moisture (%)	15·6	16·5	16·4	14·3
Calcium (%)	1·14	1·0	1·2	1·2
Magnesium (%)	0·13	0·12	0·12	0·12
Phosphorus (%)	0·81	0·79	0·78	0·74
Vitamin B_6 (mg/kg)	9·8	7·6	7·7	7·4
Vitamin D (IU/kg)	1330	1550	1250	1690
Oxalate (ppm)	400	450	450	650

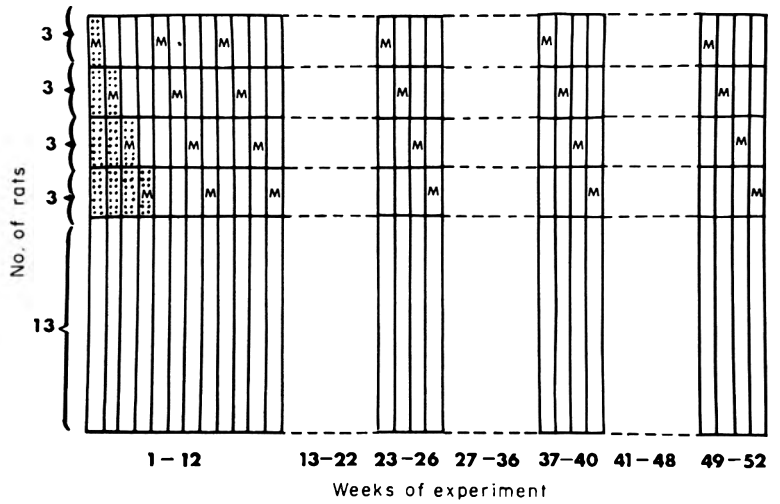

Fig. 1. Design of Experiment I for one group of 25 rats. M denotes a week spent in metabolism cages, the shaded area indicates control diet, and the unshaded area, diet appropriate to the group. The same pattern was followed for each diet group.

non-metabolism animal subsequently took its place in the metabolism cages.

Post mortem *and histopathological examination.* All rats killed, or dying during the experiment, were subjected to a thorough autopsy, using a standardized technique. The bladder and ureter were examined *in situ*, removed, fixed and prepared, with portions of kidney for routine microscopy and histochemistry. One kidney was bisected along its axis and prepared for scanning electron microscopy. At all *post mortem* examinations carried out at the end of the experiment the following organs were weighed: kidneys, lungs, heart, liver, caecum (weight with and without contents), brain, spleen, thymus, adrenals, pituitary and ovaries. These organs, along with samples of uterus, stomach, duodenum, jejunum, ileum, colon, rectum, pancreas, thyroid, parathyroid, mesenteric lymph node and any macroscopically abnormal tissues were preserved in buffered formalin. From 32 of these rats (four from each group) sections of liver, aorta, thyroid, parathyroid, caecum and ovary were stained with H & E and by von Kossa's method for mineralization. Sections of liver and aorta from all animals, abnormal tissue removed at *post mortem* and a total of 52 parathyroid glands, representing each group, were stained with H & E alone. Mineral deposits were localized by the metal substitution method of von Kossa and a number of techniques were used to demonstrate calcium in sections. A scoring system (0–5) was used to assess the amount of silver deposited (calcification) in von Kossa stained sections. Kidney sections from all treatment groups were randomized and scored for deposits localized in cortical and medullary structures. The slides were again randomized and scored for deposits localized in four sites (i) cortex (ii) medulla (iii) pelvic epithelium (iv) urinary space.

Analysis of kidney tissue for minerals. About one half of one kidney, preserved in buffered formalin, was available for analysis from each animal killed at the end of the experiment. It was removed from formalin, dried on tissue paper, transferred to a silica crucible and cut into small pieces with scissors, dried at 100 C to constant weight (48 hr) and then ashed at 550 C overnight. The ash was dissolved in 1 ml of 50% (v/v HCl, then diluted to 10 ml with water. Calcium was determined colorimetrically with cresolphthalein complexone (Morin, 1974) or by atomic absorption spectrometry, good agreement being obtained between the two methods.

Urine analysis. The pH of freshly-passed samples was determined immediately and a microscopic examination was carried out for the presence of crystals, particularly calcium oxalate, calcium phosphate, magnesium ammonium phosphate or uric acid. Oxalate was determined colorimetrically after reduction to glycollic acid with zinc (Hodgkinson & Williams, 1972). Magnesium was determined by atomic absorption spectroscopy, using a lanthanum diluent and phosphate and creatinine by automatic colorimetry. (Technicon Auto Analyzer Methods AA II-04 and AA II-11, respectively).

Statistical analysis. The significance of between-group body weight differences was determined by analysis of covariance using the initial body weight as covariate. Differences in food consumption, urine data, organ weights and calcium content of kidney tissue were investigated by analysis of variance, using logarithmic transformation of the data, if appropriate. For data on rats in the metabolism cages the analyses of variance were carried out separately for each 4-wk sojourn, the data being treated as for a 4 (groups) × 4 (weeks) design. Differences in graded histological scores were tested by the Kruskal–Wallis one-way analysis of variance by ranks (Kruskal & Wallis, 1952; Kruskal & Wallis, 1953) while mammary gland tumour incidences were compared by the method of Peto, Pike, Day *et al.* (1980).

Table 3. *Mortality*

	Control	E1414	E1422	Lactose
		Experiment I		
Initial number of rats	25	25	25	25
Unscheduled deaths (wk)	1(12)	1(9)	0	0
Interim kills	7	6	6	6
Final kills	17	18	19	19
		Experiment II		
Initial number of rats	25	25	25	25
Unscheduled deaths (wk)	2(7,32)	3(3,16,30)	2(29,31)	1(21)
Interim kills	2	2	2	2
Final kills	21	20	21	22

RESULTS

General health and mortality

No animal in either experiment showed any disturbance of behaviour. Two rats in Experiment I and eight in Experiment II died prematurely (Table 3). All deaths were due to subcutaneous tumours in the mammary glands with the exception of three animals, a control animal in Experiment I which died after 12 weeks from wasting due to unknown causes, an animal on E1414 in Experiment I which was found to have a large bladder stone consisting of magnesium ammonium phosphate at 9 wk and a control animal in Experiment II which was found dead after 7 wk from unknown causes.

Body weight

In Experiment I the mean body weight of the animals receiving lactose did not differ significantly from that of the controls but in Experiment II the mean body weight of animals on the lactose diet was significantly lower ($P < 0.01$). The mean weights of the animals receiving acetylated distarch phosphate (E1414) and acetylated distarch adipate (E1422), on the other hand, tended to be slightly higher than those of the controls, though the difference did not reach the 5% level of significance in either experiment (Fig. 2).

Food consumption

The mean daily weight of food consumed by animals while they were in the metabolism cages decreased progressively with increasing age of the animals, from about 18 g per day in weanling rats weighing 50–100 g to about 2 g per day in 18-month-old rats weighing 400–500 g. Food consumption data were not available from animals that were not in metabolism cages, for comparison, but the very low amount of food consumed towards the end of the study is considered to be artefactual and secondary to stress associated with residence in all-glass metabolism cages. No statistically significant differences between groups were seen except that in Experiment I the lactose group ate about 2 g/rat/day more than did the controls from weeks 5 to 12.

Urine analysis

The results from two of the six 4-wk periods in metabolism cages in Experiment I and for two of the three 4-wk periods in Experiment II, each a mean from 12 animals per group, are summarized in Table 4. These findings and those at the other time points (not presented for reasons of space) are summarized below.

Volume. The mean daily urine volume was appreciably higher in the animals receiving lactose than in those receiving the control diet although this was only significant in Experiment I. No significant differences were seen in animals receiving E1414 and E1422.

pH. The mean pH of freshly voided urine was reduced in rats receiving lactose compared with the control animals, this being highly significant ($P < 0.001$) in Experiment I. No significant change was observed in the animals receiving the modified starches.

Calcium. Both the mean concentration of calcium in the urine and the total daily excretion of calcium were significantly increased in the lactose group. This increase was seen at all times except weeks 1–4 in both experiments and was often very highly significant ($P < 0.001$). Increases in both calcium measures were seen at nearly all time points for both the modified starches. This increase was less marked in the E1414 group, where it was never statistically significant, and more marked in the E1422 group where a

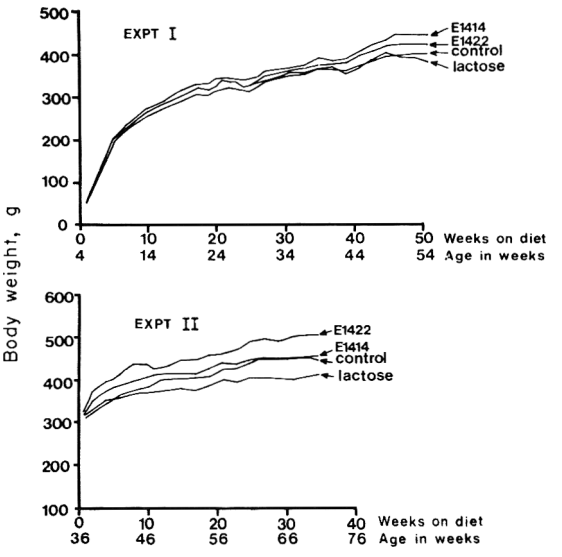

Fig. 2. Changes in the mean body weights with age and time on diet.

Table 4. *Urine composition*

	Experiment I					Experiment II				
	Weeks on diet	Control	E1414	E1422	Lactose	Weeks on diet	Control	E1414	E1422	Lactose
Volume (ml/day)	23–26	18·7	21·7	18·8	32·1*	15–18	11·2	17·5	12·7	18·0
	49–52	18·8	17·3	15·4	27·9	27–30	11·7	15·8	12·9	16·1
pH	23–26	NA	NA	NA	NA	15–18	6·76	6·45	6·71	6·24†
	49–52	9·13	7·22	7·01	6·13†††	27–30	6·93	6·76	7·04	6·55
Calcium (mmol/litre)	23–26	2·03	2·73	4·97***	6·96***	15–18	2·60	2·88	3·59	6·01***
	49–52	3·57	4·00	5·13	6·90**	27–30	3·10	2·96	4·61	6·71**
Calcium (μmol/day)	23–26	41·3	53·2	83·7***	177·8***	15–18	23·3	48·9	46·3	115·2***
	49–52	58·3	64·9	61·2	154·5***	27–30	30·2	45·0	56·0	92·7***
Phosphate (mmol/litre)	23–26	34·8	34·0	38·0	31·5	15–18	51·0	39·0	51·1	46·1
	49–52	33·5	37·4	44·3	30·0	27–30	46·5	44·2	55·3	42·6
Magnesium (mmol/litre)	23–26	7·45	8·58	12·57**	8·84	15–18	6·61	6·04	7·58	7·98
	49–52	6·48	7·13	7·78	6·73	27–30	7·67	8·09	7·80	7·86
Oxalate (mmol/litre)	23–26	2·61	3·19	2·13	1·63	15–18	1·84	1·17†	1·63	1·29
	49–52	1·73	1·25†	1·33†	1·04††	27–30	1·59	1·46	1·67	1·23
Creatinine (mmol/litre)	23–26	6·67	6·45	5·95	4·83†	15–18	11·2	7·78	10·9	7·73
	49–52	6·13	6·95	7·00	4·07††	27–30	9·53	8·02	9·70	6·82
Calcium phosphate product (mmol/litre)^2	23–26	71	96	208**	245**	15–18	151	130	189	267*
	49–52	129	153	253	243	27–30	165	137	257	312
Calcium oxalate product (mmol/litre)^2	23–26	4·23	6·60	11·5*	10·9*	15–18	4·94	3·38	5·84	7·17
	49–52	6·83	5·39	7·58	7·97	27–30	5·36	4·80	7·96	9·02

NA = Data not available

Values marked with asterisks show a significant increase and those marked with daggers a significant decrease (analysis of variance) compared with the control (* or † $P < 0{\cdot}05$; ** or †† $P < 0{\cdot}01$; *** or ††† $P < 0{\cdot}001$).

number of tests showed a significant or near significant difference.

Phosphate, oxalate, magnesium and creatinine excretion. There were only minor differences in the urinary concentrations of phosphate, oxalate, magnesium and creatinine between rats on the four diets. The oxalate and creatinine concentrations actually decreased on the lactose diet, as a consequence of the increased urine volumes. There was an increase in urinary magnesium concentration in rats fed on E1422 in both experiments but this was only significant in two cases (weeks 9–12 and 23–26 in Experiment I).

Calcium × phosphate concentration product. This measure of the tendency for calcium phosphate to precipitate from the urine was significantly raised on the lactose diet and to a lesser extent on the E1422 diet, whereas the product was not significantly changed on E1414.

Calcium × oxalate concentration product. The calcium × oxalate product tended to be higher on the lactose and E1422 diets though the differences were generally not statistically significant.

Occurrence of calcium phosphate and calcium oxalate crystals in urinary sediment. Crystals of calcium phosphate were observed in over half the samples of fresh urine collected during weeks 49–52 in Experiment I or during weeks 27–30 in Experiment II but there was no treatment-related pattern, the number of rats (out of 12) in which crystals were observed being in Experiment I: control 6, E1414 7, E1422 7 and lactose 5 and in Experiment II: control 8, E1414 5, E1422 9 and lactose 4. Crystals were also seen in a third of the samples seen during weeks 37–40 in Experiment I or weeks 15–18 in Experiment II but again there was no significant treatment effect. Calcium oxalate crystals were observed in only one (control) sample in Experiment I and in two samples (one control and one E1422) in Experiment II. Crystals of magnesium ammonium phosphate were not encountered in either of the experiments.

Analysis of faeces for minerals

No pronounced differences in faecal concentration of calcium, magnesium or phosphorus were observed between groups. Most of the differences that occurred reflected differences in food intake; when these were allowed for there was an apparent slight deficit in the faecal concentration of calcium in rats on the lactose diet, compared with the controls, but no detectable deficit for those on the E1414 or E1422 diets.

Calcium content of kidney tissue

There were considerable variations between individual animals but calcium concentrations were nearly all higher in the lactose-treated animals compared with the controls in Experiment I, the mean concentration (22·9 μmol/g dry weight of tissue) being 2·4 times higher than that of the controls and the difference was highly significant ($P < 0.001$). The mean concentrations in the E1414 and E1422-treated animals were also significantly ($P < 0.01$) higher than that of the controls, though the increase was not as pronounced as in the case of lactose (Table 5). The results for the older rats of Experiment II followed a similar pattern to that for Experiment I except that values, including those in the controls, tended to be higher than in the younger animals and a significant excess was seen only in the lactose group ($P < 0.05$).

Organ weights

All three treated groups in both experiments exhibited caecal enlargement which was most marked and highly significant for the lactose group (Table 6). The lactose group also had raised relative liver weights, raised relative and absolute adrenal weights, reduced relative and absolute thymus weights, raised relative kidney weights and raised relative and absolute spleen weights in both experiments though the increases for the kidney and spleen were not significant in Experiment I. The E1422 group showed a significant increase in absolute weight of a number of organs (kidneys, liver, heart, spleen) in Experiment II but in this experiment the total rat weight was also increased in this group. When relative weights were considered, where significant differences existed between the E1414 or E1422 groups and the controls, these did not seem indicative of toxicological effect since there was no consistent pattern for the two experiments and the weight changes were not associated with any detectable histopathological changes.

Histological findings in the kidney

Calcium deposits were observed in four different sites:

(i) in the cortex, deposited in the basement membrane surrounding the tubules or in the interstitial space,
(ii) in the medulla, as intratubular and extratubular deposits,
(iii) within the cells of the normal or hyperplastic epithelium lining the pelvic urinary space or deposited beneath it, or,
(iv) in some sections, as casts and amorphous deposits within the pelvic urinary space.

The cortico-medullary pattern of calcification typical of hyper-vitaminosis D or of magnesium deficiency was not encountered in either experiment. The degree of calcification based upon a subjective system of scoring for rats killed at termination is summarized in

Table 5. *Calcium content of kidney tissue from chemical analysis*

	Kidney calcium level (μmol g dry wt)*			
	Control	E1414	E1422	Lactose
Experiment I	9·4 ± 0·5 (17)	14·7 ± 1·9 (18)	13·2 ± 1·3 (19)	22·9 ± 2·8 (19)
Experiment II	25·3 ± 3·9 (21)	34·7 ± 7·4 (20)	33·7 ± 4 (21)	59·3 ± 10·6 (22)

*Values are means ± SEM for the numbers of animals given in parentheses.

Table 6. *Absolute and relative organ weights*

	Absolute organ weights (g)				Relative organ weights (g/100 g body wt)			
	Control	E1414	E1422	Lactose	Control	E1414	E1422	Lactose
				Experiment I				
Caecum and contents	4·98	6·28***	6·27**	10·16***	1·20	1·38*	1·51*	2·54***
Caecum empty	1·49	1·67*	1·77**	2·44***	0·36	0·37	0·42*	0·61***
Kidneys	3·46	3·44	3·43	3·68	0·84	0·76	0·80	0·92
Liver	15·15	16·16	15·95	15·96	3·67	3·54	3·66	3·96
Heart	1·32	1·33	1·34	1·30	0·32	0·29	0·31	0·33
Brain	1·95	1·92	1·92	1·93	0·48	0·42††	0·45	0·49
Adrenal	0·08	0·08	0·09	0·14††	0·021	0·018	0·022	0·035†
Spleen	0·58	0·57	0·62	0·65	0·14	0·13	0·14	0·16
Thymus	0·42	0·42	0·42	0·26††	0·10	0·09	0·09	0·06†††
				Experiment II				
Caecum and contents	6·30	7·39**	9·18***	10·71***	1·40	1·61	1·77**	2·56***
Caecum empty	1·86	2·09*	2·59***	2·73***	0·41	0·45	0·49*	0·66***
Kidneys	3·16	3·43*	3·50**	3·34	0·69	0·74	0·67	0·80**
Liver	15·11	16·55	18·07***	15·98	3·29	3·54*	3·42	3·82***
Heart	1·32	1·44*	1·50***	1·32	0·29	0·31	0·29	0·32
Brain	2·02	1·91†	1·97	1·98	0·45	0·42	0·38†	0·48
Adrenal	0·09	0·10	0·09	0·10*	0·020	0·021	0·017	0·024***
Spleen	0·53	0·59*	0·69***	0·70***	0·12	0·13	0·13	0·17***
Thymus	0·44	0·36	0·62	0·28††	0·09	0·07	0·11	0·07††

Values marked with asterisks show a significant increase and those marked with daggers a significant decrease (analysis of variance) compared with the control (* or † $P < 0·05$; ** or †† $P < 0·01$; *** or ††† $P < 0·001$).

Table 7. Compared with rats in the control, E1414 and E1422 groups mineral deposits of all the kinds listed above were most prominent in the lactose group, with significant excesses in Experiment I for the pelvic epithelium ($P < 0·01$) and in Experiment II for the cortex ($P < 0·001$), pelvic epithelium ($P < 0·001$) and urinary space ($P < 0·05$). There was also a general tendency for rats in the E1414 and E1422 groups to show greater deposition than those in the control groups but this was less marked than for lactose and only statistically significant ($P < 0·05$) in the case of the urinary space for the E1422 group in Experiment II. Calcification associated with the pelvic epithelium and urinary space, as illustrated in Figs 3 and 4, represents a change that has, in recent years, been referred to as pelvic nephrocalcinosis (PN). Thus, all three treatments were associated with PN but lactose had more effect than either of the two modified starches.

Histopathological findings in other tissues

All organs and tissues exhibiting macroscopic changes and in addition the samples taken of bladder, ureters, liver, aorta, parathyroids, caecum and ovaries were examined microscopically. No mineral deposits were found in the bladders or ureters.

Table 7. *Calcification of the kidney in rats killed at termination determined by histological assessment*

		Mean calcification score* in the			
Diet	No. of rats	Cortex	Medulla	Pelvic epithelium	Urinary space
			Experiment I		
Control	17	0·18	0·06	0·65	0·06
E1414	18	0·28	0·22	1·28	0·94
E1422	19	0·26	0·26	1·42	0·63
Lactose	19	0·37	0·26	2·59	0·95
			Experiment II		
Control	21	1·48	0·33	0·38	0·24
E1414	20	1·15	0·40	0·85	0·45
E1422	21	0·91	0·95	1·05	0·67
Lactose	22	2·68	1·05	2·46	1·91

*The calcification scores were attributed as follows: 1 = trace, 2 = small deposits, 3 = small generalized deposits, 4 = larger generalized deposits, 5 = large deposits.

Table 8. *Incidence of fatty change in the liver of rats killed at termination*

Group	No. of animals examined	Incidence of fatty change: No change	1 Minimal	2 Slight	3 Moderate	4 Severe	Any change (%)	Mean score
Experiment I								
Control	16	14	2	0	0	0	12·5	0·13
E1414	18	12	1	4	1	0	33·3	0·67
E1422	15	11	2	2	0	0	26·7	0·40
Lactose	19	19	0	0	0	0	0·0	0·00
Experiment II								
Control	21	14	4	2	1	0	33·3	0·52
E1414	20	15	4	1	0	0	25·0	0·30
E1422	21	12	1	5	3	0	42·9	0·95
Lactose	21	21	0	0	0	0	0·0	0·00

The incidence of fatty change in the liver is summarized in Table 8. Although there was no significant difference between incidence in the control, E1414 and E1422 groups, this incidence was significantly higher than the zero incidence seen in the lactose group in both experiments (Experiment I, $P < 0.05$; Experiment II, ($P < 0.01$).

Aortic calcification was not seen in Experiment I but was seen in Experiment II in all groups. No difference was seen between the mean scores in the control, E1414 and E1422 groups, but there was a highly significant increase ($P < 0.001$) in the lactose group (Table 9).

Focal hyperplasia of the parathyroid was seen in two rats in Experiment II, one a control and one a lactose animal and fibrosis was seen in the parathyroid of one rat of the E1414 group in Experiment II. There was therefore no treatment-related excess of hypertrophy or neoplasia to account for the excessive calcification seen in lactose-treated rats and to a lesser extent in rats exposed to E1414 or E1422.

No histological abnormality was seen in samples of caeca (four from each group in each experiment) despite the macroscopically visible enlargement. Inflammation of the caecum in one E1422 rat of Experiment II was not considered to be related to treatment. Similarly, enlargement and necrosis of the ovary of a lactose-fed rat in Experiment I and various other lesions seen in tissues exhibiting macroscopic changes (Experiment I, E1422, endometrial polyp; Experiment II, E1414, cystic degeneration of adrenal; E1422, pituitary adenomas in two rats) were thought to be attributable to background pathology.

Mammary gland tumours

Mammary gland tumours were observed in 23 animals (Table 10). All except four tumours were examined histologically and all were found to be fibroadenomas. Although the incidence of these tumours was slightly lower in the controls than in other groups, there was no statistically significant between-group difference, using Peto's method of analysis ($\chi^2 = 4.6$ on 3 d.f., $P < 0.1$). This type of tumour is a common feature of *ad lib.*-fed laboratory rats (Roe, 1979).

DISCUSSION

The two modified starches we have examined appear to have similar, but less marked effects to those of lactose in causing caecal enlargement and in increasing absorption and excretion of calcium. Caecal enlargement is probably due to an increased amount of osmotically-active material in the intestine which, in turn, may be caused by a failure to digest, or absorb completely the increased quantity of dietary carbohydrate before the food bolus reaches the caecum (Leegwater, de Groot & van Kalmthout-Kuyper, 1974; Walker, 1978). The hypercalciuria which occurs after the ingestion of lactose is secondary to increased intestinal absorption of calcium, though the precise mechanism is still not clear (Norman, Morawski & Fordtran, 1980; Pansu, Bellaton & Bronner, 1979).

Various types of renal calcification (nephrocalcinosis) have been described in the rat. These include (1) cortical calcification, seen after giving ip injections of calcium salts sufficient to cause hypercalcaemia and

Table 9. *Incidence of aortic calcification in Experiment II in rats killed at termination*

Group	No. of animals examined	Incidence of aortic calcification: No change	1 Minimal	2 Slight	3 Moderate	4 Severe	Any change (%)	Mean score
Control	18	8	2	4	4	0	55·6	1·22
E1414	19	7	3	2	5	2	63·2	1·58
E1422	21	10	2	5	2	2	52·4	1·24
Lactose	19	3	0	5	3	8	84·2	2·68

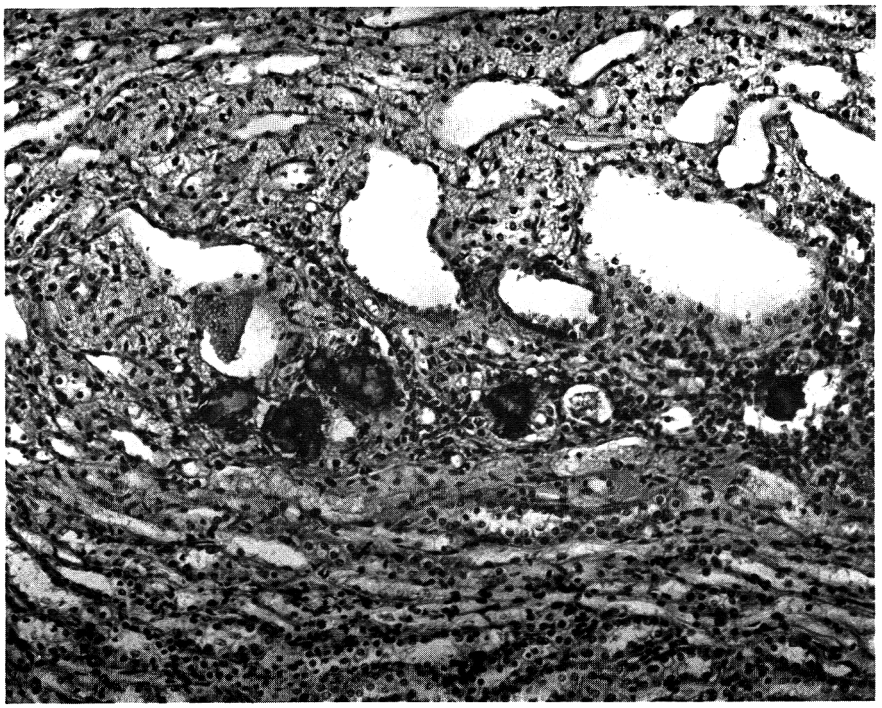

Fig. 3. Pelvic nephrocalcinosis. Epithelial hyperplasia, adhesions and mineral deposits in a recess of the pelvic fornix from a 75-wk-old control rat. Experiment II, H & E × 80.

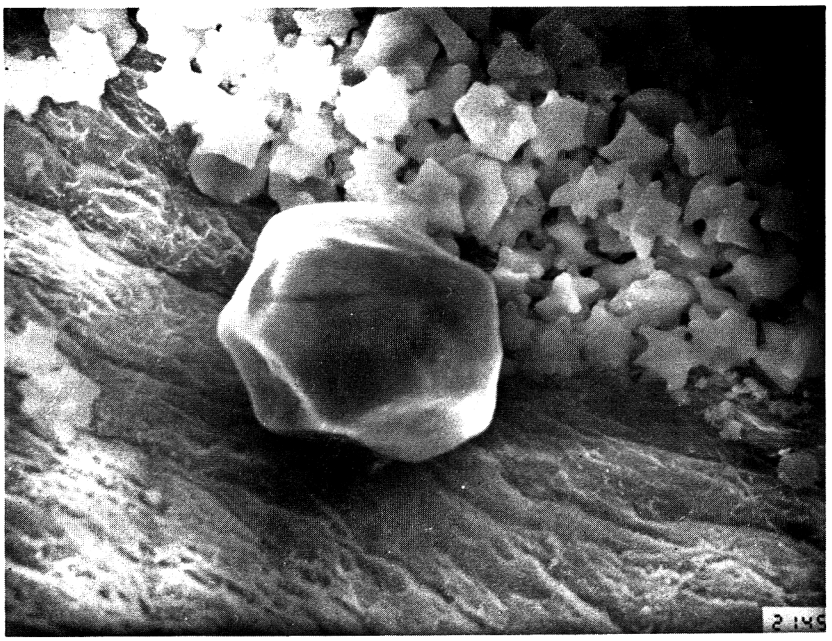

Fig. 4. Crenated red blood cells and a mineral deposit lying on epithelium lining the urinary space of the pelvic fornix. S.E.M. × 3000.

Table 10. *Incidence of mammary gland tumours*

	Incidence of mammary tumours			
Diet...	Control	E1414	E1422	Lactose
Experiment I				
Unscheduled deaths	0/1	0/1	0/0	0/0
Interim	1/7	0/6	1/6	2/6
Final kills	0/17	1/18	3/19	1/19
Total	1/25	1/25	4/25	3/25
Experiment II				
Unscheduled deaths	1/2	3/3	2/2	1/1
Interim kills	0/2	0/2	0/2	0/2
Final kills	0/21	3/20	2/21	2/22
Total	1/25	6/25	4/25	3/25
Experiments I & II combined				
Observed tumours	2	7	8	6
Expected tumours	5·7	5·6	5·8	5·9

hypercalciuria (Fourman, 1959) (2) cortico-medullary calcification, such as occurs after administering parathyroid hormone, vitamin D or inorganic phosphate (Fourman, 1959; Holdsworth & Hodgkinson, 1961) or under conditions of dietary magnesium deficiency (Du Bruyn, 1972; Schneeberger & Morrison, 1965); (3) a more recently recognized calcification in the pelvic region, particularly in the calyx, usually accompanied by calcified deposits in the pelvic lumen (pelvic nephrocalcinosis) (Casey, Ayers & Robinson, 1978). All three types of calcification have been observed in rats fed high lactose diets but the pelvic type is generally predominant (de Groot & Feron, 1975/ Feron *et al.* 1978; Sambhavaphol, Bosworth & McCay, 1958).

The two modified starches had similar, but less marked, effects to those of lactose, the effects generally decreasing in the order: lactose > E1422 > E1414. There was no evidence that lactose or either of the modified starches had any effect on any parameter other than caecal weight and mineral metabolism.

The extent of the effect of chemically-modified starches on mineral metabolism appears to be related to the type of chemical substitution, the dose-level and possibly the degree of substitution of the starch (Chen, Tsai & Nesheim, 1980). The more substituted starch used in this study (E1422; DS = 0·08) had, in the case of most of the measured parameters, more effect on mineral metabolism than the less substituted preparation (E1414; DS = 0·05), but this difference could be due to differing effects of phosphate and adipate substitution rather than to variations in the degree of substitution.

The data summarized in Tables 5 and 7 show that the calcium content of the kidneys was higher in older rats (Experiment II) than in younger rats on the same diet (Experiment I) even for rats on the control diet. This increase could be due to an excessive intake of calcium or phosphate since the concentrations in the diet were both about twice those currently recommended for optimum growth, gestation or lactation by the National Academy of Sciences (1978). These high intakes were chosen deliberately to make the present results comparable with those of previous workers but further information is clearly required regarding the optimum concentrations of calcium and phosphorus for use in long-term toxicity studies. The calcium content of the kidney might well be a useful index for monitoring studies of this kind.

Acknowledgements—The authors wish to thank Mr P. N. Lee for statistical advice, Dr W. F. Vogel and Mr E. G. Rapp for helpful comments and Mr Neil Crossley for expert technical assistance.

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MUTAGENICITY TESTING IN THE *SALMONELLA TYPHIMURIUM* ASSAY OF PHENOLIC COMPOUNDS AND PHENOLIC FRACTIONS OBTAINED FROM SMOKEHOUSE SMOKE CONDENSATES

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(*Received 6 October 1981*)

Abstract—Smokehouse smoke, which is used for flavouring meat products, was investigated for its mutagenic activity in the *Salmonella typhimurium* assay. We were chiefly concerned with the fractions free of polycyclic aromatic hydrocarbons but containing phenol compounds, which are responsible for the preservative and aromatizing properties of the smoke. The most abundantly occurring phenol compounds (phenol, cresols, 2,4-dimethylphenol, brenzcatechine, syringol, eugenol, vanilline and guaiacol) gave negative results when they were tested for mutagenicity at five concentrations up to 5000 μg/plate, with and without S-9 mix, using five strains of *S. typhimurium*. Even when phenol was further investigated in a variety of test conditions, no induction of his^+ revertants was observed. When smokehouse smoke was condensed and fractionated the majority of the various phenolic fractions also gave negative results when tested at five concentrations using five strains of *S. typhimurium*. However there was a slight increase in the number of revertants in a few cases. The presence in the phenolic fractions of very small amounts of mutagenic impurities, the nature of which needs further investigation, cannot be excluded. These results support the further development of non-hazardous smoke-aroma preparations, based on the phenolic components of smokehouse smoke.

INTRODUCTION

In the Federal Republic of Germany, a relatively large number of foodstuffs are subjected to smoking for preservative and aromatization purposes. The type of wood used to generate the smoke is mainly dependent on the aroma required. The only restriction is that the wood should remain natural and not be treated with glues, paints, impregnating or other preparations. During the process of smoking, the woods are first ignited and then allowed to smoulder at temperatures of 400–500 C, although higher temperatures up to 800 C may also be used (Toth, 1980). The resulting smoke contains at least 300 different compounds, mainly phenols, carbonyls, acids, furans, alcohols, esters, lactones as well as PAHs (Hamm, 1977; Möhler, 1978). The proportions of the constituents depends on the type of wood used and the process of smoke production. The PAHs are, however, well known for their mutagenic and carcinogenic properties (reviewed in: Brookes, 1977; Eisenbrand & Wiessler, 1981). Therefore, much effort is being directed towards the elimination of PAHs from the smoke aromas to avoid unnecessary contamination of food products. Since the preservative and aromatizing properties of the smoke are mainly attributable to the phenol-containing fractions, the enrichment of this fraction is one of the first goals in the development of non-hazardous smoke aroma preparations.

The genetic toxicology data on phenols, derivatives of phenol or even on the phenolic fractions from the smoke condensates are sparse or unavailable (Dean, 1978). Some phenols have been shown to exert carcinogenic or cocarcinogenic effects in mice (Boutwell & Bosch, 1959) and rats (Boyland, Busby, Dukes *et al.* 1964) after topical application. The most elaborate mutagenicity study was performed by Levan & Tjio (1948a,b) who observed that 40 different phenols and related compounds were weak inducers of chromosome fragmentation. Only benzene, which is thought to be metabolized to phenol, (Cited in Dean, 1978), rescorcinol, phenol, cresols, 2,4-dimethylphenol and eugenol (Florin, Rutberg, Curval & Enzell, 1980; Gilbert, Rondelet, Poncelet & Mercier, 1980; Gocke, King, Eckhardt & Wild, 1980; McCann, Choi, Yamasaki & Ames, 1975; Shahin, Bugaut, Gilard & Kalopissis, 1980) have been tested in the standard Ames system using histidine-auxotrophic *Salmonella typhimurium* strains, with negative results (except in the study by Gocke *et al.* (1980) who found phenol to be mutagenic towards *S. typhimurium* TA98).

No other data on the genetic toxicology of the phenol compounds found in the smoke or in the smoke condensates is available. We therefore investigated phenolic smoke fractions and isolated phenols for their potential mutagenicity in the Ames *S. typhimurium* assay. This test is well known for its sensitive detection of mutagens and therefore possible carcinogens (McCann & Ames, 1976). The aim was to provide preliminary information on the possible biological activity of this class of compounds and to aid their evaluation (Pool, 1980).

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Abbreviations: PAH = Polycyclic aromatic hydrocarbon

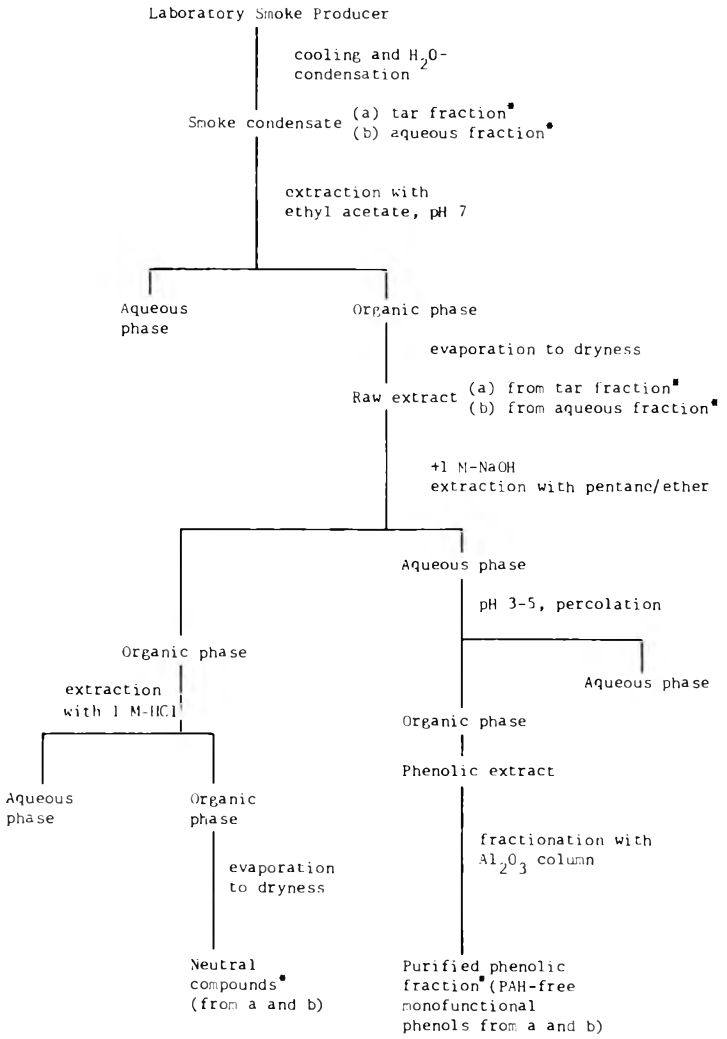

Fig. 1. Preparation of fractions containing neutral compounds and monofunctional phenols from smokehouse smoke condensate. Fractions indicated by asterisks were tested for mutagenicity.

EXPERIMENTAL

Preparation of test samples. The PAH-free phenolic fractions to be tested for mutagenicity were prepared and kindly supplied by L. Toth, Kulmbach, according to the schemes in Figs 1 & 2 (Toth, 1980). Smokehouse smoke was produced by slowly burning 200 g birchwood spans at 600°C in a laboratory smoke producer. From this, two smoke condensates, a tar fraction (a) and an aqueous fraction (b), were obtained through condensation by water scrubbing. Further extraction of the condensates with ethyl acetate or through perforation yielded two raw extracts (from (a) and (b) respectively) which were ultimately purified as shown in Figs 1 & 2. Extraction with NaOH yields organic and aqueous phases (Fig. 1) which are further purified to yield neutral compounds and monofunctional phenols, respectively. This method destroys the bifunctional phenolic compounds, therefore an additional purification scheme was employed. For isolation of the mono- and bifunctional phenolic compounds, (Fig. 2) the raw extracts were purified by steam distillation at 170°C and partition chromatography on silanized silica gel. This phenolic extract was subjected to capillary gas chromatography after which more than 100 different phenolic compounds were found and partly identified.

The ten most abundant constituents of the purified phenolic fraction of birchwood smoke are phenol (1·95%), *o*-cresol (0·86%), *m*-cresol (0·77%), *p*-cresol (0·53%), 2,6-dimethylphenol (0·22%), guaiacol (9·06%), brenzcatechine (1·85%), syringol (9·42%), eugenol (1·31%) and vanilline (0·42%). Phenol, *m*-cresol, *p*-cresol and vanilline were supplied by Schuchardt, Munich, *o*-cresol and eugenol came from Merck, Darmstadt, 2,4-dimethylphenol guaiacol and brenzcatechine from Fluka, Buchs, CH and the syringol from EGA-Chemie, Steinbach. They were all either 98% pure or of analytical grade except for 2,4-dimethylphenol which was 90% pure and contained 5–7% dimethylcresol as well as traces of cresols.

Mutagenicity testing. The histidine auxotrophic indicator strains *Salmonella typhimurium* TA1535, TA1537, TA1538, TA98 and TA100 were kindly sup-

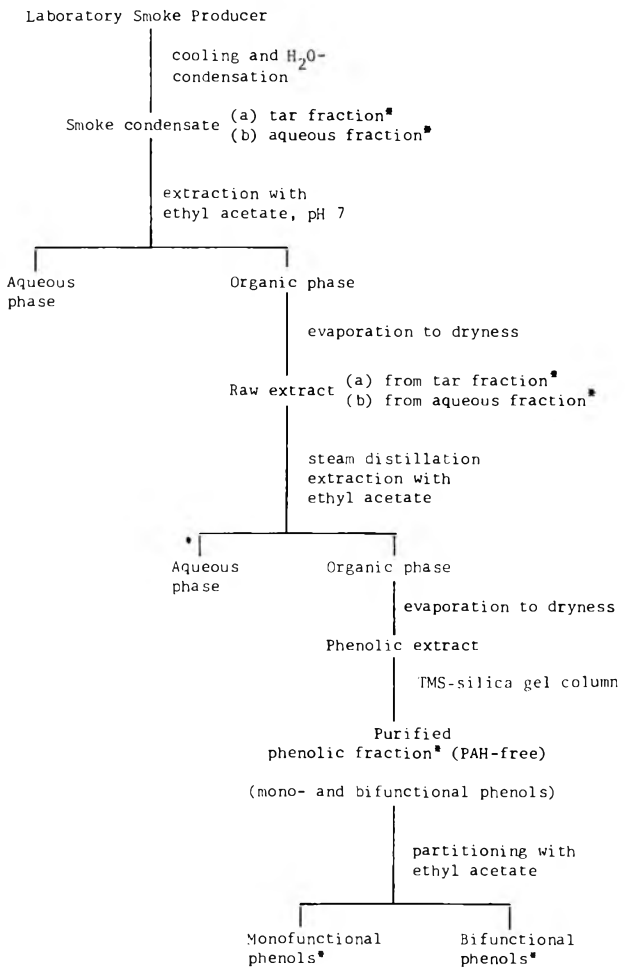

Fig. 2. Preparation of fractions containing mono and bifunctional phenols from smokehouse smoke condensate. Fractions indicated by asterisks were tested for mutagenicity.

plied by Dr. B. N. Ames, California. The test for their reversion to histidine prototrophy was performed using the plate incorporation assay as previously described (Ames, McCann & Yamasaki, 1975). Each compound or smoke condensate fraction was dissolved in DMSO and tested in five concentrations in quadruplicate in the presence of buffer pH 7·4 or S-9 mix with cofactors (60 mg S-9 protein/10 ml S-9 mix) from Aroclor-pretreated Sprague-Dawley rats. The S-9 protein content was determined using the biuret-method-kit of Boehringer, Mannheim. The sensitivity of the S-9 and of the indicator strains was monitored routinely using positive control compounds during each experiment. Table 2 summarizes the combined positive control data obtained in the individual experiments. Significance levels for positive dose-response effects were obtained with the Joncheere test (Hollander & Wolfe, 1973).

Special procedures in addition to the standard method were used to study the mutagenicity of the parent compound, phenol. These included incorporation of S-9 from untreated Sprague-Dawley rats, preincubation of the compound (added in 100 μl H_2O) with the bacteria and S-9 mix prior to plating, addition of S-9 mix with various protein contents, and inclusion of the comutagen norharman during the test.

RESULTS

The mutagenicity of the ten phenolic compounds detected in smoke condensates were investigated in the Ames Test (Table 1). Each compound was tested in quadruplicate at five concentrations in each of the five bacterial strains, both in the presence and absence of the S-9 mix. The number of histidine revertants scored in the presence of the test compounds phenol, *o*-, *m*-, *p*-cresol, 2,4-dimethylphenol, branzcatechine, guaiacol, syringol, vanilline and eugenol, never more than slightly exceeded that number of spontaneously arising revertants. For all of the compounds the only dose-related effect was toxicity at the highest tested concentration (5000 μg). This was apparent as a thinning of the bacterial background lawn and as a reduced number of spontaneous revertants.

Phenol was further tested using a variety of experimental modifications with *S. typhimurium* TA98 as indicator organism. The test was carried out using S-9 from untreated Sprague-Dawley rats to determine whether Aroclor-induced detoxifying enzymes played

Table 1. *Non-mutagenicity of phenolic compounds isolated from the PAH-free phenolic fraction of smoke condensate*

Compound	Concentration (μg/plate)	No. of his$^+$ revertants/plate									
		TA1535		TA1537		TA1538		TA98		TA100	
		−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9
Phenol	0	28	12	16	14	12	18	15	27	89	76
	0·5	32	20	16	11	12	19	19	19	103	65
	5	32	14	17	16	11	18	19	26	100	82
	50	30	19	14	14	13	21	23	30	101	85
	500	28	14	16	23	11	22	23	31	103	76
	5000*	0	0	9*	0	8	16	8	21	21	49
o-Cresol	0	22	12	10	14	17	30	21	35	89	108
	5	14	11	6	7	12	29	26	35	100	84
	50	16	11	9	9	13	16	21	33	107	90
	500	30	11	7	4	14	20	22	36	100	90
	5000*	0	0	0	0	1	11	0	14	98	30
m-Cresol	0	12	9	5	11	8	18	16	14	91	76
	0·5	22	11	5	NT	9	22	17	16	NT	91
	5	21	4	6	17	11	20	19	14	117	86
	50	23	2*	8	13	7	29	18	14	62	85
	500	15	9*	8	16	7	20	15	17	53	97
	5000*	0	2	0	0	4	4	3	2	0	32
p-Cresol	0	12	22	5	11	16	36	21	23	67	76
	0·5	22	31	10	11	16	30	20	30	65	90
	5	24	31	3	NT	21	34	18	31	68	81
	50	21	26	10	10	14	38	20	31	67	87
	500	21	29	6	11	15	37	18	29	64	100
	5000*	26	0	0	0	7	20	29	9	3	12
2,4-Dimethylphenol	0	11	12	12	9	8	20	16	41	89	76
	0·5	17	12	5	5	10	18	21	12	106	65
	5	11	15	7	12	6	20	21	20	100	82
	50	13	22	3	5	7	20	20	20	102	85
	500	15	17	6	4	6	19	17	14	87	76
	5000*	0	0	0	0	0	0	0	1	15	49

Brenzcatechine	0	20	18	16	15	17	23	12	22	67	65
	0·5	16	17	13	17	18	25	19	20	71	67
	5	16	16	15	17	14	23	21	22	65	65
	50	17	20	17	17	13	26	19	23	63	69
	500	16	24	15	15	13	28	17	27	68	68
	5000*	2	14	3	17	11	19	9	23	29	63
Guaiacol	0	24	9	5	14	5	10	19	19	67	76
	0·5	21	16	7	12	13	12	20	34	66	94
	5	26	13	7	16	9	11	18	36	71	93
	50	22	19	4	13	10	11	23	14	65	81
	500	15	16	4	9	14	9	22	16	67	95
	5000*	25	9	0	13	13	10	16	5	57	64
Syringol	0	11	12	12	9	16	6	9	41	67	76
	0·5	9	16	6	9	16	10	8	22	66	88
	5	18	16	3	10	19	5	8	25	67	77
	50	12	12	8	6	16	9	11	24	71	71
	500	10	14	8	12	16	10	13	25	65	77
	5000*	0	0	0	0	9	4	4	10	61	79
Vanilline	0	22	12	16	14	17	18	27	41	67	76
	0·5	13	19	22	14	16	22	18	39	66	78
	5	14	11	15	7	17	22	20	15	68	81
	50	39	11	17	14	16	22	20	11	65	76
	500	28	17	15	9	10	20	12	10	63	62
	5000*	0	0	13	0	1	6	0	7	0	38
Eugenol	0	15	18	10	15	15	23	17	22	91	65
	0·5	11	17	9	20	18	20	16	21	79	65
	5	11	16	8	16	18	26	15	28	77	66
	50	12	20	9	17	16	21	15	24	77	71
	500	14	18	9	16	16	22	6	20	100	57
	5000*	0	0	0	0	0	0	0	0	0	0
Positive controls											
Sodium azide	2	526 ± 54	—	—	—	—	—	—	—	502 ± 28	—
2-Nitrofluorene	3	—	—	—	—	284 ± 60	—	275 ± 74	—	—	—
9-Aminoacridine	60	—	—	810 ± 95	—	—	—	—	—	—	—
2-Aminoanthracene	5	—	306 ± 60	—	269 ± 47	—	1027 ± 199	—	1093 ± 227	—	723 ± 159

NT = Not tested

*These concentrations resulted in toxicity which was apparent as a thinning of the background lawn.

All of the values for test compounds are means of four plates and those for negative controls are means of eight plates. In all cases the standard deviation was within 5–10% of the mean. The positive control values are means ± SD from 7–15 independent experiments.

Table 2. *Mutagenicity testing of phenol using* Salmonella typhimurium *TA98 and S-9 from induced and uninduced Sprague-Dawley rats*

Concentration		No. of his$^+$ revertants/plate	
(μmol/plate)	(μg/plate)	With S-9 from Aroclor induced rats	With S-9 from untreated rats
0	0	22	31
10	940	19	31
20	1880	28	35
30	2820	33	26
40	3760	30	29
50	4700	30	33
60	5640*	32	18
70	6580	28	23
80	7520	22	20
90	8460	16	16
100	9400	17	18

*This concentration resulted in toxicity which was apparent as a thinning of the background lawn.

Values are means of four plates except for the control values which are means of eight plates. In all cases standard deviation was within 5–10% of the mean.

Table 3. *Mutagenicity of phenol towards* Salmonella typhimurium *TA98 in the presence of varying amounts of Aroclor-induced S-9 mix. Preincubation was carried out prior to plating (see experimental)*

Phenol concentration		S-9 protein	No. of his$^+$ revertants/plate			
μmol/plate)	(μg/plate)	(mg/10 ml S-9 mix)...	20	60	100	
0	0		36	32	31	41
10	940		39	39	34	30
20	1880		35	43	37	19
30*	2820		0	0	34	0
40	3760		0	0	0	0

*This concentration resulted in toxicity which was apparent as a thinning of the background lawn.

Values are means of three plates except for the control values which are means of eight plates. In all cases the standard deviation was within 5–10% of the mean.

Table 4. *Effect of norharman (200 μg/plate) on the mutagenicity of phenol in* Salmonella typhimurium *TA98 after preincubation with S-9 mix containing different protein concentrations*

		No. of his$^+$ revertants/plate					
	S-9 protein (mg/10 ml S-9 mix)...	20		40		60	
Concentration (μg/plate)	Norharman	–	+	–	+	–	+
0		20	20	21	21	20	20
0·5		17	16	16	18	21	18
5		16	18	19	19	22	19
50		17	15	20	19	23	17
500		15	15	20	17	18	17
5000*		15	9	17	10	15	8

*This concentration resulted in toxicity which was apparent as a thinning of the background lawn.

Values are means of three plates.

Table 5. *Mutagenicity testing of crude and purified smoke condensate-fractions containing phenols*

Fraction	Concentration (μg/plate)	No of his$^+$ revertants/plate									
		TA1535		TA1537		TA1538		TA98		TA100	
		−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9
Smoke condensate	0	16	16	13	11	18	22	16	32	76	113
(tar fraction	0·5	20	21	11	13	18	25	19	36	66	96
Figs 1 and 2)	5	20	21	13	23	20	34	20	36	69	102
	50	19	24	13	18	17	28	16	38	69	107
	500	17	15	13	23	14	30	19	0	65	103
	5000†	0	12	0	17	0	29	0	0	0	96
Smoke condensate	0	16	18	13	12	18	20	16	29	76	108
(aqueous fraction;	0·5	16	19	13	15	15	25	18	28	64	111
Figs 1 and 2)	5	16	19	15	11	15	26	17	21	63	98
	50	21	20	11	13	15	23	14	27	69	104
	500	17	19	14	10	14	20	14	0	77	104
	5000†	0	21	0	13	0	18	0	0	0	108
Raw-extract	0	19	22	6	9	14	27	33	47	145	110
(from tar fraction;	0·5	25	16	7	14	20	21	34	48	121	125
Figs 1 and 2)	5	24	20	9	11	11	30	27	46	146	78
	50	21	24	5	12	14	31	23	32	123	113
	500	24	16	8	7	20	26	24	43	154	136
	5000†	0	13	0	9	0	0	0	21	0	90
Raw-extract	0	21	23	14	22	23	33	19	43	127	163
(from aqueous fraction;	0·5	29	16	14	25	22	30	18	38	109	153
Figs 1 and 2)	5	27	26	14	20	19	34	20	36	121	157
	50	29	26	20	21	18	32	21	39	131	134
	500	27	19	18	24	27	32	25	33	144	142
	5000†	0	18	0	13	0	26	0	30	17	152
Neutral compounds	0	9	10	9	6	8	13	19	18	163	97
(Fig. 1)	0·5	7	12	8	9	5	15	13	13	176	103
	5	8	9	4	7	2	15	11	14	161	101
	50	6	7	4	7	3	16	8	19	165	101
	500	3	7	8	7	3	15	9	14	189	94
	5000†	4	7	0	10	0	19	0	52*	229*	108
Monofunctional	0	7	13	9	6	8	14	19	20	174	120
phenols (Fig. 1)	0·5	7	9	6	11	6	11	17	19	151	112
	5	9	10	4	6	7	18	19	14	157	117
	50	7	13	3	7	8	13	17	20	172	100
	500	6	9	2	10	10	12	24	14	177	109
	5000†	0	6	0	6	1	11	0	16	108	120
Purified phenolic	0	17	13	9	10	14	18	17	171	178	
fraction (Fig. 2)	0·5	21	13	8	7	16	20	19	23	152	143
	5	20	15	8	5	16	22	20	24	168	149
	50	19	12	8	5	15	20	21	23	175	150
	500	18	16	6	5	14	17	7	23	209*	152
	5000†	4	15	4	6	6	12	0	17	0	150
Monofunctional	0	7	13	9	6	8	14	19	20	177	103
phenols (Fig. 2)	0·5	7	9	6	11	6	11	17	17	178	104
	5	9	10	4	6	7	18	19	14	176	101
	50	7	13	3	7	8	13	17	20	184	99
	500	6	9	2	10	10	12	24	14	204	101
	5000†	0	6	0	6	1	11	0	16	236*	86
Bifunctional	0	9	10	9	6	8	13	19	18	163	97
phenols (Fig. 2)	0·5	8	11	11	6	7	12	11	19	152	99
	5	7	10	8	5	7	12	11	17	157	86
	50	8	9	6	6	9	11	9	15	162	75
	500	10	6	7	6	8	12	7	15	167	71
	5000†	7	5	5	5	1	12	0	13	194*	74

†These values resulted in toxicity which was apparent as a thinning of the background lawn.

Values are means of four plates except the control values which are means of eight plates. In all cases the standard deviation was within 5–10% of the mean. Values marked with an asterisk show a significant dose-related increase (Joncheere test) in the number of revertants ($P < 0{\cdot}01$).

a role in masking a mutagenic effect of phenol. Table 2 shows that for the concentration range shown (0–9400 μg/plate), no mutagenic activity was detected.

When the procedure included preincubation of phenol with the bacteria for 30 min at room temperature in the presence of S-9 mix with various protein concentrations no phenol-induced effect was observed, other than increased toxicity at much lower doses than was apparent from the plate incorporation assay (Table 3). Table 4 shows that even after including the

Table 6. *Mutagenicity testing of high concentrations of three smoke condensate fractions in* Salmonella typhimurium

mix) Concentration of fraction (μg/plate)	Strain... / Fraction...	No. of his$^+$ revertants/plate: TA100 (without S-9 mix) Neutral compounds	Monofunctional phenols	Bifunctional phenols	TA98 (with S-9 mix) Neutral compounds
0		108	101	108	29‡
500		117	114	102	47
1000		120	130	100	49
2500		132	145	100	62
5000		166	156	109	76
7500		183	147*	110	84
10000		160*	0†	124	96
20000		0†	0	147*	106

†These concentrations resulted in toxicity which was apparent as a thinning of the background lawn.

Values are means for three plates except for that indicated by a double dagger which is the mean of six plates. Those values marked with an asterisk show a significant dose-related increase (Joncheere test) in the number of revertants ($P < 0.01$).

comutagen norharman, using different amounts of S-9 mix, no mutagenic effect of phenol is observable.

The possible mutagenic activity of the crude and purified phenol-containing fractions described in Figs 1 & 2 was investigated. Table 5 gives the results of testing one fraction of each type. Results with all of the fractions isolated were confirmed in at least a second experiment. In almost all of the tests the numbers of induced his$^+$ revertants did not reach a doubling of the numbers of spontaneous his$^+$ revertants. The crudest smoke condensates were also tested at 1000 μg/plate in the presence of S-9 because of their high toxicity at 5000 μg but this also produced negative results. A suspicion of possible mutagenic activity was observed in TA100, without S-9 for some of the more purified fractions containing neural and phenolic compounds. Further experiments (Table 6) at higher concentrations showed a slight increase in the number of his$^+$ revertants in a dose-related manner up to 5000–20,000 μg/plate. These increases were significant ($P < 0.01$), as determined by the nonparametric Joncheere test, a multisample procedure to test against ordered alternatives (increasing doses; Hollander & Wolfe, 1973). For the neutral compounds a further distinct mutagenic effect was observed with S-9 using TA98 (Table 6).

DISCUSSION AND CONCLUSIONS

The ten most abundant phenolic compounds found in the phenolic fractions that may be used in smoke flavourings for meat products did not show any mutagenicity in the Ames assay under standard conditions. Even when testing the parent compound, phenol, using a variety of modifications of the standard system, no enhancement of the number of his$^+$ revertants was observed. These modifications, including the use of S-9 from uninduced rats, performing a preincubation prior to plating or varying the protein content of the S-9 mix (Ames *et al.* 1975; Bartsch, Malaveille, Camus *et al.* 1980) are often necessary to detect otherwise weakly active mutagens by offering more sensitive test conditions. The comutagenic activity of norharman has also been described (Nagao, Yahagi, Kawachi *et al.* 1977) and its use has been proposed to aid the detection of some mutagenic compounds evading detection under standard conditions. However its use did not enhance the activity of phenol. These results are in agreement with those of Shahin *et al.* (1980) and Gilbert *et al.* (1980) who also failed to detect any mutagenic activity of phenol in the *S. typhimurium* system, but are in contradiction to results published by Gocke *et al.* (1980). These authors used a different agar for expression of his$^+$ revertants and counted *c.* 34 TA98-his$^+$ revertants at 50 μmol phenol compared with 14 for the control. Whether this low 'activity' which is based on the low number of spontaneous revertants in this strain is a sign of actual genetic activity of the compound remains to be clarified and could be further evaluated using a more sensitive indicator organism and test system.

The smoke condensates and their separate fractions also produced largely negative results. Smoke condensate does include mutagenic polycyclic aromatic hydrocarbons, which may, however evade detection because of their low concentrations in the crude extract. A slight enhancement of mutagenic events was recorded in *S. typhimurium* TA100 for the neutral compounds (purified according to Fig. 1), as well as for mono and bifunctional phenolic compounds of the phenol extract (purified according to Fig. 2). This slight enhancement of his$^+$ revertant yield (which never reached a doubling of the number of spontaneous revertants, but did increase in a dose-related manner) was confirmed when the experiment was repeated using higher concentrations of the test samples. Whether or not we are dealing with traces of true mutagenic compounds, in the fractions, which are inducing reversion of the tester bacteria, must be

further clarified. In addition to the most abundantly occurring phenols listed above, Toth (1980) found small amounts of over 100 additional compounds with phenolic properties present in the phenolic extract tested here. Furthermore Gilbert *et al.* (1980) found *p*-nitrosophenol to be a direct acting mutagen in TA1538. It is therefore conceivable that the slight enhancement may be due to trace compounds such as nitroso derivatives, to other impurities, or to the combined effects of the compounds within the mixture. These suspected mutagenic trace compounds in the phenolic fraction are probably only enriched during the purification procedure shown in Fig. 2, but not during the process shown in Fig. 1. The monofunctional phenols obtained according to the latter scheme do not contain detectable mutagenic impurities. The phenolic fractions are free of polycyclic aromatic hydrocarbons (or at least their levels are below the limit of detection) although it is possible that they contain other neutral compounds. The fractions are known to comprise *c.* 95% phenolic compounds and 5% unidentified compounds. An attempt to clarify the nature of the possible mutagens in the mono and dihydroxy fractions of Fig. 2 is presently being made (L. Toth, personal communication, 1981).

The neutral compounds were also distinctly mutagenic towards TA98 after metabolic activation with S-9 mix. This fraction includes polycyclic aromatic hydrocarbons which are known mutagens in this system. The neutral compounds are probably not essential flavouring components of the smoke aroma preparations.

The most abundant phenolic compounds found in smokehouse smoke condensates were not mutagenic in the *S. typhimurium* assay. The presence of mutagenic impurities in the phenolic fractions needs further investigation as does the value of the phenolic fractions as flavouring preparations. These mutagenicity data alone are not sufficient for risk assessment. Further genotoxicity and other toxicity data are needed for an evaluation of the potential hazard to man of the phenolic compounds (Pool, 1981; Scientific Committee of the Food Safety Council, 1980). Nevertheless, the results are promising for the further development of smoke flavour preparations free of polycyclic aromatic hydrocarbons.

Acknowledgements—This work was supported by the Deutsche Foschungsgemeinschaft Grant no. Schm 81/18. We thank Dr L. Toth from the Institute of Chemistry and Physics at the Federal Agency for Meat Research, Kulmbach, FRG for valuable discussions and for preparing and supplying all the samples used in this work. We are grateful to Mr R. Gliniorz and Mrs R. Zimmermann for excellent technical assistance.

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Fd Chem. Toxic. Vol. 20, pp. 393 to 399, 1982
Printed in Great Britain

0278-6915/82/040393-07$03.00/0
Pergamon Press Ltd

DOSE-RESPONSE STUDIES IN CARCINOGENESIS BY NITROSO-*N*-METHYL-*N*-(2-PHENYL)ETHYLAMINE IN RATS AND THE EFFECTS OF DEUTERIUM SUBSTITUTION*

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(*Received 11 December 1981*)

Abstract—A dose-response study of the carcinogenicity of nitroso-*N*-methyl-*N*-(2-phenyl)ethylamine was carried out in male Fischer 344 rats. The compound was given in drinking water at concentrations of 115, 28, 9·5, 3·2, 1·1 and 0·4 mg/litre. The highest concentration proved toxic leading to the early death of several animals; the remainder of this group were treated for 21 wk. All of the other concentrations were given for 33 wk, except the 28 mg/litre treatment which ceased at 30 wk. An additional group of rats was given 0·4 mg/litre for 104 wk. In all groups of animals, except those in the high-dose group that died early and those given 0·4 mg/litre for 33 wk, 50% or more of the animals had tumours of the oesophagus or forestomach or both when they died. In several groups the number of rats with these tumours approached 100%. The total dose of carcinogen received by the rats in the lowest dose group was 1·3 mg and 45% of them had tumours of the upper gastro-intestinal tract. The effect on carcinogenicity of labelling the nitrosamine with deuterium in either the methyl group or the α-methylene of the phenylethyl group was determined by treating groups of rats with equimolar concentrations of the deuterium-labelled and unlabelled nitrosamine. A very significant increase in carcinogenic effectiveness was observed with the compound containing deuterium in the α-methylene of the phenylethyl group, suggesting that methylation might not be the important event in carcinogenesis by this compound in rats.

INTRODUCTION

The effect of deuterium substitution for hydrogen in nitrosamines has usually been to reduce carcinogenic potency when the deuterium is in the α position to the nitroso group. This was so for nitrosodimethylamine (Keefer, Lijinsky & Garcia, 1973), nitrosomorpholine (Lijinsky, Taylor & Keefer, 1976), nitrosoazetidine (Lijinsky & Taylor, 1977) and nitroso-2,6-dimethylmorpholine (Lijinsky, Saavedra, Reuber & Blackwell, 1980). However, in the asymmetric nitrosomethylethylamine (NMEA), deuterium in the α-methyl group reduced carcinogenicity while deuterium in the α position of the ethyl group increased carcinogenicity (Lijinsky & Reuber, 1980). To establish whether this effect was unique to NMEA, we conducted a similar study with the asymmetric nitroso-*N*-methyl-*N*-(2-phenyl)ethylamine (NMPEA) a derivative of NMEA. Since several dose levels of the deuterium-labelled NMPEAs were to be used to establish the possible potency differences, we decided to extend the study to considerably lower doses of the unlabelled compound in order to investigate the relationship between tumour response and dose in the Fischer rat. NMPEA was reported to be a potent inducer of tumours of the oesophagus in BD rats (Druckrey, Preussmann, Ivankovic & Schmähl, 1967) and was found to have the same target in Fischer rats.

*This work was supported by Contract No. NO1-CO-75380, with the National Cancer Institute, NIH, Bethesda, Maryland 20014.

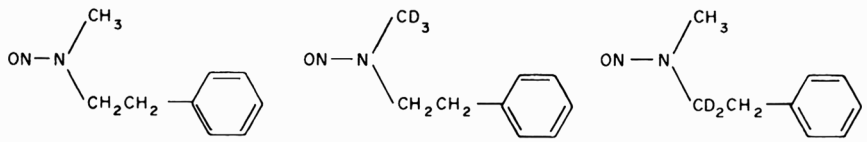
Abbreviations NMEA = Nitrosomethylethylamine; NMPEA = Nitroso-*N*-methyl-*N*-(2-phenyl)ethylamine; [*Me*-D]NMPEA = Nitroso-*N*-[D_3]methyl-*N*-(2-phenyl)ethylamine; [*Et*-D]NMPEA = Nitroso-*N*-methyl-*N*-(2-phenyl)-[1-D_2]ethylamine.

EXPERIMENTAL

Chemicals

NMPEA. A solution of methylphenylethylamine, 25 g (0·185 mol), in 50 ml of concentrated hydrochloric acid was cooled to 5°C. A solution of 25·5 g (0·37 mol) of sodium nitrite in 60 ml of distilled water was added dropwise. When addition was complete the ice-bath was removed and the mixture was stirred at 25°C for 3 hr. The nitrosamine was extracted into dichloromethane, and washed with 5% sodium bicarbonate solution. The organic layer was dried over anhydrous potassium carbonate, filtered and the solvent removed on a rotary evaporator. The residual oil was vacuum distilled giving 20·82 g (69%) of NMPEA (Fig. 1a): b.p. 174–176°C at 15 mmHg; IR (film) 3300, 3015, 1600 cm^{-1}; λ_{max}(EtOH) 341 nm (ε 87); n.m.r. ($CDCl_3$) δ 2·84 (s, 2·22) syn, (CH_3) δ 3·38 (s, 0·78H) anti (CH_3); δ 4·15 (t) anti; δ 3·60 (t) syn, (2H); δ 7·18 (m, 5H); *m/z* (%) 164 (8), 134 (28), 105 (16), 104 (83), 91 (100), 73 (28), 43 (42), 42 (63). Analysis found: C, 65·76%; H, 7·32%; N, 17·09%; calculated for $C_9H_{12}N_2O$: C, 65·85%; H, 7·32%; N 17·07%.

*Nitroso-*N*-[D_3]methyl-N-(2-phenyl)ethylamine* ([Me-*D*]*NMPEA*). A mixture of 13·4 g (0·081 mol) of nitroso-*N*-methyl-*N*-(2-phenyl)ethylamine in 2 g of sodium deuteroxide and 40 ml of deuterium oxide was



(a) (b) (c)

Fig. 1. Structures of nitroso-*N*-methyl-*N*-(2-phenyl)ethylamine (a) and its deuterium-labelled derivatives, nitroso-*N*-[D_3]methyl-*N*-(2-phenyl)ethylamine (b) and nitroso-*N*-methyl-*N*-(2-phenyl)-[1-D_2]-ethylamine (c).

refluxed for 12 hr with stirring. The mixture was extracted with dichloromethane. The solvent was removed, and the deuteration process was repeated four times. The mixture was extracted with dichloromethane, dried over sodium sulphate and the solvent was removed by rotary evaporation. Distillation of the residual oil under vacuum gave 8·4 g (62%) of [*Me*-D]NMPEA (Fig. 1b): 176–178°C at 15 mmHg; λ_{max} (EtOH) 341 (ϵ 88); n.m.r. ($CDCl_3$), δ 2·65 (m, 2H); δ 4·15 anti (t, 1·5H); δ 3·60 syn (t, 0·5H); δ 7·25 (s, 5H); MS (70 eV), *m/z* (%), 167 (1), 136 (4), 104 (24), 91 (100), 65 (18), 45 (14). Analysis found: C, 64·74%; H and D, 7·35%; N, 16·79%; calculated for $C_9H_9D_3N_2O$: C, 64·64%; H and D, 7·19%; N 16·75%.

N-*Methyl*-(*2-phenyl*)*acetamide*. Methylamine hydrochloride (13·4 g; 0·2 mol) was dissolved in 200 ml of ice-cold 10% aqueous sodium hydroxide solution. To the cold solution was added 20 g (0·129 mol) of phenyl acetyl chloride dropwise. The cooling bath was removed and the reaction mixture was stirred at 25°C for 2 hr. The crude amide was extracted into methylene chloride, dried over potassium carbonate and filtered through a layer of magnesium sulphate. The solvent was evaporated and the crude product was recrystallized from light petroleum ether:tetrahydrofuran to give 18·4 g (95·7%) of amide: m.p. 57–59°C; IR ($CHCl_3$) 3445, 3345, 3090, 3060, 1660, 1600, 1550 cm^{-1}; n.m.r. ($CDCl_3$) δ 2·76 (d, 3H); δ 3·59 (s, 2H); δ 5·4 (b, 1H); δ 7·3 (m, 5H).

N-*Methyl*-N-(*2-phenyl*)-[*1-*D_2]*ethylamine*. A 250-ml flask equipped with a soxhlet extractor and a condenser was flame dried and cooled with nitrogen. The flask was charged with 8·4 g (0·2 mol) of lithium aluminium deuteride, and 114 ml of anhydrous ether and 17 g (0·114 mol) of *N*-methyl-(2-phenyl)acetamide in a glass thimble. The mixture was refluxed until all the amide had been dissolved (1 hr), further refluxed for 4 hr and then stirred at room temperature overnight. The mixture was cooled to 5°C, hydrolysed with 50 ml of cold deuterium oxide. Sodium sulphate was added to the hydrolysed mixture and this was followed by filtration through a layer of magnesium sulphate. The solvent was removed on a rotary evaporator and the residue was vacuum distilled to yield 4 g (26%) of product: b.p. 105°C at 25 mmHg; IR (film) 3300, 3015, 2600, 2170, 1600 cm^{-1}; n.m.r. ($CDCl_3$) δ 7·24 (m, 5H); δ 2·41 (s, 3H); δ 1·54 (s, 1H); δ 1·80 (b, 2·3H).

Nitroso-N-*methyl*-N-(*2-phenyl*)-[*1-*D_2]*ethylamine* ([Et-*D*]*NMPEA*). To a solution of 4 g (0·038 mol) *N*-methyl-*N*-(2-phenyl-[1-D_2]ethylamine in 5 ml 12 N-hydrochloric acid and 10 ml of water at 5°C was added 3·45 g (0·05 mol) of solid sodium nitrite in small lots over a period of 1 hr. The separated yellow oil was extracted into ether, dried over anhydrous potassium carbonate. The solution was filtered, the ether removed on a rotary evaporator and the residue vacuum-distilled to give 3·9 g (79%) of [*Et*-D]-NMPEA (Fig. 1c): b.p. 84°C at 0·6 mmHg; λ_{max} (EtOH) 341 nm (ϵ 87); n.m.r. ($CDCl_3$) δ 3·56 (s, 0·78H) anti CH_3; δ 2·99 (s, 2·22H) syn CH_3; δ 2·8 (b, 1·48H); δ 3·04 (b, 0·52H) δ 7·26 (m, 5H); IR (film) 3030 cm^{-1}, 2110, 1602, 1500, 1435; MS, *m/z* (%), 166 (2), 136 (13), 102 (45), 91 (100), 75 (12). Analysis found: C, 64·70%; H and D, 7·45%; N, 16·43%; calculated for $C_9H_{10}D_2N_2O$: C 65·03%, H and D 7·23%, N 16·85%.

Animal treatments

Groups of 20 male Fischer 344 rats, 6 to 8 wk old, were obtained from the colony of the Frederick Cancer Research Facility. They were housed four to a cage, which was plastic with a wire mesh bottom. The animals were given Rockland rat diet *ad lib*. The treatments consisted of 20 ml day rat (80 ml cage) of a solution of NMPEA or one of its deuterium-labelled derivatives as drinking water, 5 days wk. Almost all of the solutions were drunk by the animals and there was little spillage. In case the animals suffered a water deficit, on the remaining 2 days of each week tap water was supplied *ad lib*. Each solution supplied to the animals was prepared by dilution of a concentrated stock solution in ethanol prepared approximately every 3 months and stored. There was no detectable change in the composition of the stock solutions, which were kept in the dark. Treatment of each group of animals continued for a specified number of weeks, after which they were allowed to die naturally and were given complete autopsy, and all lesions and major organs, with or without lesions, were fixed for histological examination. Seven of the animals in the untreated control group and one each in two of the low-dose groups were killed at the end of the study.

Statistical methods

The survival patterns for the several test groups are compared both graphically and analytically. The graphs of the survival curves are based on the Kaplan–Meier methodology (Kaplan & Meier, 1958) which adjusts for censored observations. The only censored observations resulted from the relatively few animals that survived to the end of the study. The individual survival curves were compared, pairwise, using the chi-square test of Mantel & Haenszel (1959). The analyses were produced with the computer program developed and described by Thomas, Breslow & Gart (1977).

Final tumour incidences were analysed for those

dose-groups where survival patterns did not preclude meaningful comparisons, i.e., either no statistical difference betwen survival curves was detected, or, if a statistical difference between survival curves was detected, the group with the shortened life-span also displayed a higher tumour incidence. The comparisons of the control group with a single dosed group were made with the one-sided Fisher exact test (Fisher, 1946). The Cochran–Armitage test (Armitage, 1971) was used to test for positive trend; one-sided probabilities are given.

RESULTS

The complete results of histopathological examination of all the rats in the study, group by group, are given in Table 1. A very large proportion of the rats

Table 1. *Results of histopathological examination of rats given labelled and unlabelled NMPEA in the drinking water at various dose levels*

Test compound	NMPEA							[*Me*-D]NMPEA				[*Et*-D]NMPEA		
Group No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Concentration (mg/l)	115	28	9·5	3·2	1·1	0·4	0·4	117	29	10	3·3	9·8	3·3	0
Duration of treatment (wk)	21	30	33	33	33	33	104	21	30	33	33	33	33	—
Total dose (mg/rat)	242	84	31	19·5	3·6	1·3	4·1	246	87	33	11	32	11	0
	No. of animals with tumours													
Total tumours of upper GI tract														
Carcinomas	5	18	17	16	9	6	7	9	17	17	18	15	17	0
Carcinomas and/or papillomas	9	19	19	16	11	9	10	13	18	20	20	20	18	0
Adrenal														
Cortical carcinoma						1								1
Cortical hyperplastic nodule					2						1			
Medullary haemangioma					1									
Phaeochromocytoma				1	5	3	3				1		1	7
Anus														
Adenoma				1										
Bone marrow														
Early leukaemia														1
Monocytic leukaemia											1			
Colon														
Leiomyosarcoma					1									
Epiglottis														
Basal-cell carcinoma													1	
Oesophagus														
Basal-cell carcinoma	5	16	13	15	2	1	3	9	16	14	14	15	14	
Basal-cell papilloma	6	12	8	4	2	1	4	11	8	9	11	4	6	
Papilloma												1		
Squamous-cell carcinoma		2	3	1			1		1	5		1		
Heart														
Endocardial sarcoma							1							
Ileum														
Adenocarcinoma				1										
Liver														
Adenoma										1				
Early leukaemia							1				1	1	3	2
Haemangioma					2									1
Hepatocellular carcinoma			1		1	4	1				1			
Hyperplastic nodule					5		1				1			
Lymphangioma				1	3	2					2			
Lung														
Adenocarcinoma					2									
Carcinoma							1							
Haemangioma					1									
Metastatic adrenal carcinoma						1								
Metastatic chordoma				1										
Metastatic neurosarcoma											1			
Squamous cell carcinoma		1												
Lymph node														
Metastatic C cell carcinoma														1
Mammary gland														
Adenoma														1
Carcinoma					1	1								1
Fibroadenoma						1	1							1
Fibroadenoma—focal carcinoma														1
Fibroma					2	1	1				2			1
Leiomyoma					1									
Multiple organs														
Monocytic leukaemia				9	13	16	10				8		1	9

[*contd*]

Table 1—*continued*

Test compound	NMPEA							[*Me*-D]NMPEA				[*Et*-D]NMPEA		
Group No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Concentration (mg/l)	115	28	9·5	3·2	1·1	0·4	0·4	117	29	10	3·3	9·8	3·3	0
Duration of treatment (wk)	21	30	33	33	33	33	104	21	30	33	33	33	33	—
Total dose (mg/rat)	242	84	31	19·5	3·6	1·3	4·1	246	87	33	11	32	11	0
	No. of animals with tumours													
Nasal cavity														
Adenocarcinoma				1										
Nasopharynx														
Basal cell carcinoma													1	
Notochord														
Malignant chordoma				1										
Oral cavity														
Basal-cell carcinoma													1	
Squamous-cell carcinoma							1							
Oropharynx														
Basal-cell carcinoma													4	
Basal-cell papilloma													1	
Squamous cell carcinoma													1	
Pancreas														
Acinar carcinoma					1									
Acinar hyperplastic nodule				1			1							
Islet-cell adenoma				2	3	3	4				2	1		2
Islet-cell carcinoma				3	4	2	2				2			1
Mixed-cell adenoma											1			1
Haemangioma							1							
Parathyroid														
Adenoma						2	1							
Peritoneum														
Metastatic mesothelioma					1						1			3
Primary mesothelioma													1	
Pituitary														
Adenoma				1	1		2							3
Carcinoma				1	4	3	2							2
Haemangioma														1
Preputial gland														
Carcinoma						2	1							1
Skin														
Basal-cell carcinoma				1	1								1	1
Leiomyoma					1									
Leiomyosarcoma					1									
Lysoma					1									
Neurosarcoma					1	1					1			1
Squamous-cell carcinoma					1	2					1			
Spleen														
Early leukaemia							1				2	1	3	2
Haemangioma				1	1	2		1						
Haemangiosarcoma							3							
Stomach														
Basal-cell carcinoma		9	4	15	7	4	4		5	2	13	7	10	
Basal-cell papilloma	3	5	4	1	1	6	1	4	1	5	1		3	
Carcinoma		1								1				
Papilloma										1		3	1	
Squamous-cell carcinoma												1		
Testis														
Interstitial-cell adenoma				4	3	2	1				3		2	2
Interstitial-cell carcinoma				11	15	17	13				13		8	17
Mesothelioma					1	1					2		1	3
Thyroid														
C-cell adenoma														1
C-cell carcinoma				2	3	2	2							1
F-cell carcinoma					1									1
Tongue														
Basal-cell carcinoma				6			1				5	2	2	
Basal-cell papilloma							1					1	1	
Papilloma					1									
Squamous-cell carcinoma						1					1			
Urinary bladder														
Transitional-cell carcinoma							1							

treated with NMPEA developed tumours of the upper gastro-intestinal tract, as shown at the beginning of the table: excepted were those animals treated with high doses of the compound, which succumbed to lung infections. Unlike a previous dose-response study with nitrosodiethylamine (Lijinsky, Reuber & Riggs, 1981), it was not possible in this experiment to find a linear relationship between dose administered and time to death with tumours. This might have resulted from the large number of early deaths in the highest dose group.

At the highest dose selected, 1·15 mg/litre of NMPEA, there was considerable toxicity both with the unlabelled compound and with [*Me*-D]NMPEA. Several animals died within a short time of pneumonia which developed as a result of lung damage. In the animals that did not die, treatment was interrupted for 2 wk and then resumed. The animals that had survived had apparently eliminated the source of their injury and lived until tumours developed. Because these high doses were very effective, the original plan to continue the treatments for 30 wk was not carried out and animals died much earlier with tumours of the oesophagus. This was the common tumour induced by all of the treatments. All of the other dosage groups were carried out as planned and there were few intercurrent deaths. At the lowest concentrations of NMPEA, treatment was carried out for 33 wk and for longer periods, to assess the effect of continued treatment at low concentrations. Such an effect might have a bearing on risk assessment in this type of model system.

The survival patterns of the rats treated with unlabelled NMPEA are shown in Fig. 2. The animals in group 1 died rapidly, with ten of the 20 animals dying in the first 2 wk of the study. The survival curve for this group differed statistically ($P < 0{\cdot}00001$) from that of all the other groups. The survival curves for groups 2 and 3, receiving the next highest dosages, did not differ statistically from each other, but each was statistically different from all other groups

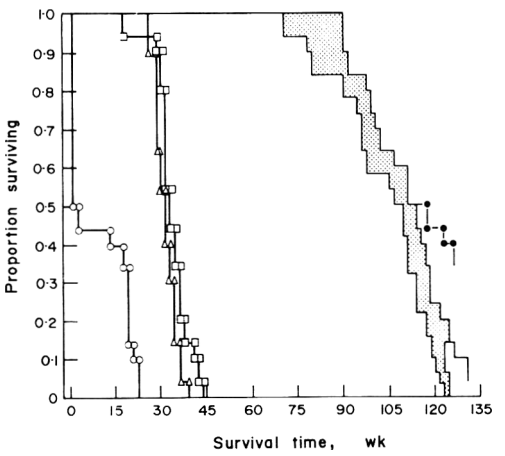


Fig. 2. Survival curves for rats given various doses of unlabelled NMPEA in the drinking water reflecting deaths from all causes. The curves for group 1 (○), group 2 (△) and group 3 (□) are individually represented. The curves for groups 4–7 lie within the shaded area. The curve for the untreated control group (●) also lies within the shaded area up to week 117.

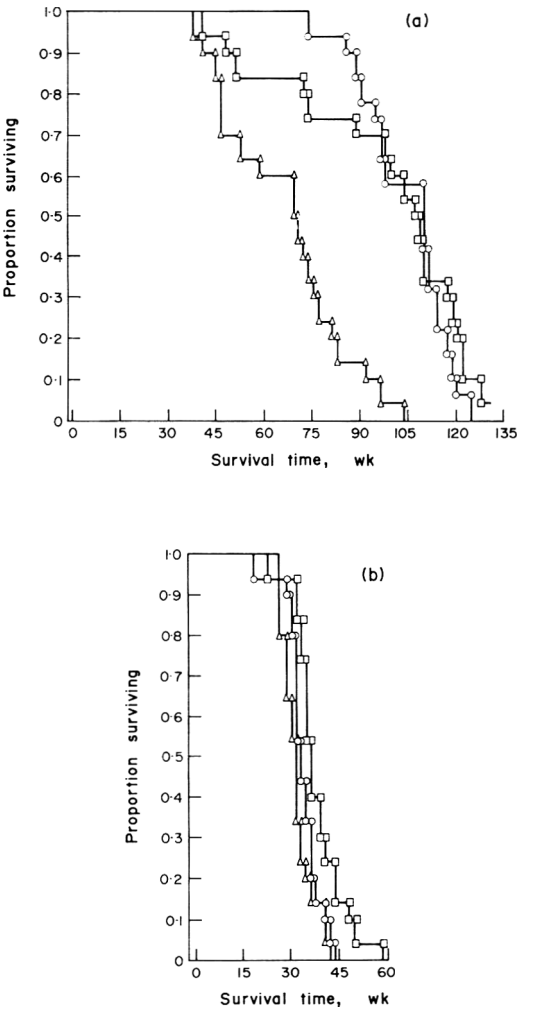


Fig. 3. Survival curves reflecting deaths from all causes of rats given (a) 3·3 mg/litre or (b) 9·5 mg/litre of unlabelled NMPEA (○), [*Me*-D]NMPEA (△) or [*Et*-D]NMPEA (□) in the drinking water.

($P < 0{\cdot}00001$). The survival curves for groups 4, 5, 6 and 7 did not differ from each other, but groups 4 and 7 differed statistically from the untreated control group ($P < 0{\cdot}05$ in each case) with the dosed animals dying sooner.

The survival curves of groups receiving comparable dosages of unlabelled and labelled NMPEA are shown in Figs 3a and 3b. Groups receiving a dose rate of 3·3 mg/litre for 33 wk are shown in Fig. 3a. The group receiving the [*Et*-D]NMPEA died sooner than the other two groups ($P < 0{\cdot}0001$). The difference in survival curves for the NMPEA and [*Me*-D]-NMPEA groups did not attain or even approach statistical significance. Fig. 3b shows the survival curves of groups receiving 9·5 to 10 mg/litre for 33 wk. Here no statistical difference exists between the unlabelled NMPEA and [*Et*-D]NMPEA groups. The survival pattern for the [*Me*-D]NMPEA group was statistically different from that of the unlabelled group ($P < 0{\cdot}05$) and that of the [*Et*-D]NMPEA group ($P < 0{\cdot}01$).

Table 2 gives the incidences for tumours of the oesophagus, forestomach and all of the upper gastro-

Table 2. *Analysis of the incidence of tumours at specific sites in male rats treated with unlabelled NMPEA at four dose rates for 33 wk*

Dose rate (mg litre) and probability†	No. and percentage of animals with tumours of the					
	Oesophagus		Forestomach		Upper GI tract*	
	Carcinoma‡	Carcinoma and or Papilloma§	Carcinoma‡	Carcinoma and or Papilloma§	Carcinoma‡	Carcinoma and or Papilloma§
0	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)
0·4	1/20 (5%)	2/20 (10%)	4/20 (20%)	8/20 (40%)	6/20 (30%)	9/20 (45%)
P	NS	NS	NS	0·0020	0·012	0·00079
1·1	2/20 (10%)	3/20 (15%)	7/20 (35%)	7/20 (35%)	9/20 (45%)	11/20 (55%)
P	NS	NS	0·0050	0·0050	0·00079	0·00010
3·2	15/20 (75%)	15/20 (75%)	15/20 (75%)	15/20 (75%)	16/20 (80%)	16/20 (80%)
P	<0·00001	<0·00001	<0·00001	<0·00001	<0·00001	<0·00001

NS = Not significant ($P \geq 0.05$)

*Animals with at least one tumour (carcinoma or papilloma) of the oesophagus, forestomach, tongue, or nasal cavity.
†The probability level for the Fisher exact test for the comparison of each treated group with the corresponding control group.
‡Animals with a carcinoma only or a carcinoma and papilloma.
§Animals in preceding column plus those with a papilloma only.

intestinal tract, the latter category including several tumours of the tongue and nasal cavity. In every case, the test for a positive dose-related trend was highly significant. In a number of cases, the incidences at the lower doses were not statistically greater than their respective controls, but, when viewed as part of a highly significant trend, the possibility that these low incidences may be compound-related cannot be overlooked.

DISCUSSION

The tumours induced were almost exclusively basal-cell papillomas and carcinomas of the oesophagus and forestomach, which have been described previously (Reuber, 1977). In many cases the tumours in the oesophagus appeared to be the cause of death of the rats. As in the previous study with nitrosodiethylamine, only in the groups of rats given the highest concentrations of NMPEA in water did the induction of tumours lead to the early death of the animals with tumours. At concentrations below 9·5 mg/litre there was little noticeable reduction of the lifespan of the animals compared with the untreated controls. Instead, the effect of higher concentrations or longer treatment times in those groups was to increase the number of animals with tumours. Both of these measures of potency were used to assess the effectiveness of treatments. It is difficult, on the basis of these results, to establish a mathematical relationship between the dose of carcinogen received by each animal and its relative risk of developing tumours, which is the objective of many mathematical treatments of carcinogenesis data. The ultimate aim is to develop equations that can be used to assess the carcinogenic risk to humans of exposure to a chemical carcinogen based on the results of a chronic toxicity test in animals, a prospect that still eludes us.

A comparison of the effectiveness in inducing tumours of the two deuterium-labelled derivatives of NMPEA shows little or no significant difference at the high dose rates of the carcinogen. This suggests that when the dose received by the animal is large, the rate of activation of NMPEA is so high that any subtle differences due to the presence of deuterium are overwhelmed by the size of the biological response. On the other hand, at lower dose rates the effectiveness of [*Et*-D]NMPEA in reducing survival time, is significantly greater than that of NMPEA itself or of [*Me*-D]NMPEA (Fig. 3a). This result is similar to that of NMEA and its α-methylene-labelled and methyl-labelled deuterium derivatives; the methylene-labelled derivative is considerably more potent than is the unlabelled compound. The 10 mg [*Me*-D]-NMPEA/litre group showed a small but significant difference in survival time (Fig. 3b) from the equivalent group treated with the unlabelled compound; the [*Me*-D]NMPEA was less active than the unlabelled compound, a result similar to that with NMEA labelled with deuterium in the methyl group, although in the latter case the effect was larger (Lijinsky & Reuber, 1980).

The results of the chronic feeding experiments with deuterium-labelled NMPEA in rats correlate well with the results of studies of the metabolism of the compounds *in vitro* using rat-liver microsomes. In these experiments (Farrelly, Stewart, Saavedra & Lijinsky, 1982) the oxidation of [*Me*-D]NMPEA to formaldehyde was reduced and that of [*Et*-D]-NMPEA to phenylacetaldehyde was reduced, compared with the unlabelled compound. The results were interpreted as indicating that phenylethylation rather than methylation by NMPEA was related to carcinogenesis. The effects of deuterium labelling in NMPEA on carcinogenicity were opposite to the effects of deuterium labelling in nitrosomethyl-*n*-butylamine, in which deuterium in the methyl group appeared to increase carcinogenicity, while deuterium in the α-methylene of the butyl group decreased carcinogenicity (Lijinsky, Reuber, Saavedra & Blackwell,

1980). The differences are unexplained, particularly in the light of the fact that both nitrosamines have the rat oesophagus as their principle target.

The notable result of the dose-response study of NMPEA is that such small doses as 4 mg total (as a solution containing 0·4 ppm NMPEA) over the lifetime of a rat can give rise to a significant incidence of tumours of the upper gastro-intestinal tract. A trend towards a significant incidence of these tumours was seen even in those animals that received only 1·3 mg during 33 wk. This is a similar sensitivity to that observed in our previous study with nitrosodiethylamine (Lijinsky, Reuber & Riggs, 1981), illustrating the remarkable sensitivity of Fischer rats to nitrosamine carcinogenesis.

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Fd Chem. Toxic. Vol. 20, pp. 401 to 406, 1982
Printed in Great Britain

0278-6915/82/040401-06$03.00/0
Pergamon Press Ltd

THE FATE OF *N*-NITROSODIETHANOLAMINE AFTER ORAL AND TOPICAL ADMINISTRATION TO RATS

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(*Received 22 December 1981*)

Abstract—^{14}C-labelled *N*-nitrosodiethanolamine ([^{14}C]NDELA) was given to Osborne–Mendel rats at two dose levels, 0·5 or 50 mg/kg, by oral or topical administration. The excreta and tissues were analysed at various times from 4 hr to 1 wk after administration to determine the distribution of radioactivity. After oral administration, [^{14}C]NDELA was rapidly absorbed from the gastro-intestinal tract, distributed throughout all organs and tissues, and then excreted, mainly via the kidneys. The tissue concentration reached a peak at 8 hr, but some activity remained after 1 wk. After topical application NDELA was slowly absorbed percutaneously, but once absorbed was distributed as in the orally dosed rats. Metabolic profiles of urine and bile samples from both the orally and topically dosed rats were identical, although the quantities varied. In addition to unchanged NDELA, one metabolite was present. The dose level had little effect on the quantities of unchanged NDELA or the metabolite present.

INTRODUCTION

N-Nitrosodiethanolamine (NDELA) has been found in many cosmetic products since 1977 (Fan, Goff, Song *et al.* 1977a). The source of the compound is believed to be due to the nitrosation of di- or triethanolamine, compounds widely used in the cosmetic industry as emulsifiers. Lijinsky, Keefer, Conrad & Van de Bogart (1972) demonstrated that triethanolamine could easily be nitrosated with appropriate nitrosating agents under mildly acidic conditions to form NDELA.

NDELA has been shown to cause liver cancers in rats after oral ingestion and neoplasms of the nasal cavity and trachea as well as liver cancers in golden hamsters after sc injections (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Hilfrich, Schmeltz & Hoffmann, 1978; Lijinsky, Reuber & Manning, 1980). NDELA belongs to a group of compounds one or more members of which have been demonstrated to be carcinogenic in all species tested so far (Wogan & Tannenbaum, 1975). Preussmann, Wurtele, Eisenbrand & Spiegelhalder (1978) reported that rats excreted unchanged in the urine up to 70% of an oral dose of 500 mg NDELA/kg. Lijinsky, Losikoff & Sansone (1981) reported that maximum skin absorption of NDELA was 78% 1 hr after application of 50 mg, and 20–30% of a 50-mg-NDELA/kg dose applied to the skin undiluted or given by gavage appeared in the urine in 24 hr.

Fan *et al.* (1977a) found that a series of lotions and shampoos contained NDELA in concentrations up to 50 ppm. They estimated human exposure to NDELA from these cosmetic products to be from 2 to 100 μg/day. NDELA has also been found in synthetic cutting fluids at very high concentrations of 0·02 to 3·0%, and could thus be a serious hazard to machinists if absorbed percutaneously (Fan, Morrison, Rounbehler *et al.* 1977b).

Percutaneous absorption is the normal route of entry of this cosmetic contaminant into the animal body; however, bioassays for carcinogenicity of suspected compounds, including NDELA, are usually conducted by the oral route. The purpose of the studies reported in this paper is to compare the disposition of NDELA in rats after oral and topical administration.

EXPERIMENTAL

Materials. Uniformly labelled [^{14}C]NDELA, specific activity 6·39 mCi/mmol, was obtained from New England Nuclear Corporation (Boston, MA); radiochemical purity was established by column chromatography. The labelled material was diluted with nonradioactive NDELA (Columbia Organic Chemicals Co., Columbia, SC) to obtain a compound of suitable activity for the metabolism study (7–8 μCi/rat). For dermal studies the compound was applied in an acetone solution, and for oral administration it was administered in an aqueous solution.

Animals and diets. Adult Osborne–Mendel rats weighing 350–400 g were used in the study. For the short-term studies (4–8 hr) the rats were maintained under phenobarbital anaesthesia for the duration of the experiment. For longer periods (24 hr to 1 wk) the rats were placed in individual glass Delmar–Roth metabolism cages designed to permit separate collections of urine, faeces and CO_2. The rats were allowed Purina ground laboratory chow (Ralston Purina Co. Inc., St Louis, MO) and water *ad lib.*

Oral administration. For the short duration experiments of 4 and 8 hr, the bile ducts of the anaesthetized male rats were cannulated by inserting a polyethylene tube. The urinary bladders were ligated at the urethra. NDELA in aqueous solution was administered by stomach tube at doses of 0·5 or 50 mg/kg body weight. The urine of each rat was withdrawn from the bladder by a syringe at the end of the experimental period. For longer periods of 1 day to 1 wk, the rats

Abbreviations: NDELA = *N*-Nitrosodiethanolamine.

were dosed in the same way but were maintained in metabolism cages; the receptacles for urine and faeces were immersed in ice during the entire collection period, and $^{14}CO_2$ was trapped in an absorption tower which contained ethylene monomethyl ether and monoethanolamine (2:1, v/v).

Dermal administration. For short-term studies (4 and 8 hr), the bile ducts were cannulated and the urethras were ligated in the same manner as for the rats treated orally. The hair was clipped from the ventral surface of an area near the neck and then [^{14}C]NDELA, as an acetone solution, was applied at 0·5 or 50 mg/kg to a skin area of 4·9 cm^2 on the clipped surface. For longer periods of 1 day to 1 wk, the [^{14}C]NDELA was applied at 0·5 or 50 mg/kg to a clipped area of 4·9 cm^2 on the dorsal surface near the back of the neck. To prevent the rats from removing and ingesting the applied compound, a restraining collar was constructed of 7/8-in. OD rubber tubing and flexible picture-hanging wire. Slits were cut along the length of the tubing to provide more flexibility. A length of wire several inches longer than the rubber tubing was run through the tubing. The collar was then placed around the trunk of the rat just behind the forelegs. The ends of the wire were attached together on the dorsal surface of the rats below the area of application. The rats remained completely mobile with this type of restrainer but were unable to reach the site of application with either their front or back paws. The animals were then placed in metabolism cages.

Distribution of [^{14}C]NDELA. At the end of each experimental period, the rats (a total of 39) were killed by immersion in solid CO_2 and the organs were immediately removed and weighed. The large organs were homogenized in water using a Brinkmann Polytron (Brinkmann Instruments, Inc., Westbury, NY). The tissue homogenates were diluted to a known volume so that a 0·1-ml aliquot from each homogenate contained 15·0 mg tissue sample. The small organs such as the endocrine glands and eyeballs were digested directly without homogenization. The 0·1-ml aliquots were put into counting vials and were allowed to dry at room temperature; then 2·0 ml Soluene (Packard Instrument Co., Downers Grove, IL) was added to each vial, and the samples were allowed to digest at room temperature. Soluene solutions of blood samples (0·05 ml) were rendered lighter in colour by adding 0·1 ml 30% hydrogen peroxide. After 30 min, 0·15 ml acetic acid was added to the vials. The carcass was digested in 600 ml saturated NaOH. After digestion the volume was measured, and a 0·1-ml aliquot was transferred to a counting vial.

Analysis of excreta. The faeces were homogenized with water and diluted to a known volume with water. Aliquots of 0·1 ml were placed in counting vials and allowed to dry at room temperature; then the samples were digested in 2 ml Soluene. Aliquots of 0·1 ml urine and bile were measured directly into counting vials. Aliquots of 2 ml from the CO_2 trap were transferred to counting vials for ^{14}C measurements.

Radioactivity measurements. All samples were assayed in a liquid scintillation spectrometer (Tri-Carb model 3375, Packard Instrument Co.). Fluor solution (15 ml) containing 0·4% of 2,5-diphenyloxazole (PPO) and 0·01% of 1,4-bis(phenyloxazoyl) benzene (POPOP) in methanol–toluene (1:3, v/v) was added to the vials containing the digested biological materials. Each sample was corrected for quenching by external standardization, and the results were expressed as percentage of dose present in each tissue or organ.

Isolation of urinary and biliary metabolites. Approximately 60 g Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ) was suspended in water and then packed into a glass column, and the column was washed with 400–500 ml water. The final bed dimensions were 65 × 1·8 cm. Urine samples from the distribution study as well as urine from three female rats dosed in the same way were used for the metabolic profile study. After application of 2 ml urine or bile, the column was eluted by gravity with water at a flow rate of 30 ml/hr. Fractions of 5 ml were collected, and ^{14}C activity was measured in each fraction.

DNA experiment. Two rats were given by stomach tube 600 mg [^{14}C]NDELA (in aqueous solution)/kg body weight. After 4 hr, the rats were killed by decapitation, and the livers were immediately removed and frozen on dry ice. DNA was isolated by a combination extraction, centrifugation, and enzymatic method (R. H. Reitz, Dow Chemical Co., Midland, MI, personal communication, 1979). The isolated DNA was hydrolysed with 2 ml 0·1 N–HCl for 1 hr at 100°C. The hydrolysate was chromatographed on a Sephadex G-10 column (68 × 1·8 cm) and eluted with 0·05 M-ammonium formate (Pegg, 1977). Each fraction was transferred to a counting vial and evaporated to dryness. After the addition of 15 ml of phosphor solution, radioactivity was measured in the samples.

RESULTS

Distribution and excretion of [^{14}C]NDELA

The results of the study of excretion and distribution of [^{14}C]NDELA administered orally at doses of 0·5 and 50 mg/kg are given in Tables 1 and 2, respectively. NDELA was rapidly absorbed from the gastrointestinal tract and distributed throughout the entire body. The highest percentage of the dose, with the exception of the gastro-intestinal tract and kidneys during the first few hours, was found in the liver at all time intervals from 4 hr to 1 wk with the peak concentration at 8 hr. Traces of ^{14}C remained 1 wk after both the single low and high doses in all organs and tissues analysed. The major route of elimination was via the kidneys. The percentage of the dose excreted in the urine increased from 20% after 4 hr to 93% after 1 wk at the 0·5-mg/kg dose and from 30 to 94% at the 50-mg/kg dose. At both levels, up to 1·5% of the dose appeared in the bile at 8 hr. After 1 wk $^{14}CO_2$ accounted for 2·0% of the dose at both dose levels, and 3·5% of the low dose and 5·1% of the high dose were eliminated in the faeces.

The results of the studies of the topically dosed animals are given in Tables 3 and 4. Some radioactivity was found in all organs at 4 hr, although most of the applied compound remained at the site of administration. The highest levels of ^{14}C were found at 96 hr and 1 wk. Urinary excretion was low during the first 24 hr but increased and 46% of the high dose and 55% of the low dose were eliminated after 1 wk.

Table 1. *The distribution of ^{14}C activity in male rats following a single oral dose of 0·5 mg [^{14}C]N-nitrosodiethanolamine/kg body weight*

Tissue	No. of rats...	^{14}C activity (% of dose) after					
		4 hr	8 hr	24 hr	48 hr	96 hr	168 hr
		2	2	2	1	1	2
Stomach (+ contents)		30·9	4·6	0·2	0·01	0·02	0·01
Small intestine (+ contents)		2·6	1·9	0·2	0·08	0·05	0·05
Large intestine (+ contents)		2·7	0·8	1·0	0·1	0·08	0·03
Liver		1·1	1·7	0·3	0·2	0·3	0·3
Kidneys		0·5	0·8	0·04	0·02	0·04	0·06
Brain		0·1	0·1	<0·01	NT	0·01	0·01
Adrenals		0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Pancreas		0·04	0·2	0·01	<0·01	<0·01	<0·01
Lungs		0·15	0·3	0·02	0·01	0·02	0·01
Salivary glands		0·05	0·04	0·01	<0·01	0·01	<0·01
Heart		0·4	0·07	<0·01	<0·01	<0·01	0·01
Spleen		0·03	0·02	<0·01	<0·01	<0·01	<0·01
Urinary bladder		0·6	0·3	0·01	<0·01	<0·01	<0·01
Eye		0·02	0·02	<0·01	<0·01	<0·01	<0·01
Testes		0·3	0·3	0·01	<0·01	0·02	0·02
Thymus		0·1	0·02	<0·01	<0·01	<0·01	<0·01
Thyroid		0·05	0·01	<0·01	<0·01	<0.01	<0.01
Pituitary		<0·01	<0·01	<0·01	<0·01	<0.01	<0.01
Carcass		12·3	35·2	1·4	0·6	1·4	1·3
Faeces		NT	NT	0·6	1·5	2·6	3·5
Urine		20·3	30·3	68·6	68·8	103·9	93·2
Bile		0·6	1·5	NT	NT	NT	NT
Expired CO_2		NT	NT	1·1	1·5	2·3	2·0

NT = Not tested

Values are means for the number of rats indicated.

Table 2. *The distribution of ^{14}C activity in male rats following a single oral dose of 50 mg [^{14}C]N-nitrosodiethanolamine/kg body weight*

Tissue	No. of rats...	^{14}C activity (% of dose) after					
		4 hr	8 hr	24 hr	48 hr	96 hr	168 hr
		1	2	1	1	1	3
Stomach (+ contents)		41·7	26·5	0·03	0·08	0·01	0·01
Small intestine (+ contents)		1·8	2·0	0·2	0·05	0·03	0·03
Large intestine (+ contents)		0·6	0·8	0·2	0·1	0·03	0·03
Liver		0·8	1·3	0·5	0·3	0·2	0·1
Kidneys		0·4	1·8	0·04	0·02	0·02	0·02
Brain		0·06	0·01	0·01	0·01	0·01	<0·01
Adrenals		0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Pancreas		0·1	0·1	<0·01	<0·01	0·01	<0·01
Lungs		0·08	0·2	0·01	0·01	0·01	0·02
Salivary glands		0·03	0·06	<0·01	<0·01	0·01	<0·01
Heart		0·05	0·1	0·01	<0·01	<0·01	0·01
Spleen		0·03	0·05	<0·01	<0·01	<0·01	<0·01
Urinary bladder		0·2	1·3	<0·01	<0·01	<0·01	<0·01
Eye		0·01	0·01	<0·01	<0·01	<0·01	<0·01
Testes		0·2	0·3	0·01	0·01	0·01	0·01
Thymus		0·02	0·05	<0·01	<0·01	<0·01	<0·01
Thyroid		0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Pituitary		<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Carcass		17·2	24·6	1·7	2·3	0·9	0·9
Faeces		NT	NT	4·2	4·6	2·2	5·1
Urine		30·3	29·7	87·1	88·2	93·4	93·6
Bile		0·05	0·7	NT	NT	NT	NT
Expired CO_2		NT	NT	1·3	0·9	1·8	2·0

NT = Not tested

Values are means for the number of rats indicated.

Table 3. *The distribution of ^{14}C activity in male rats following a single topical dose of 0·5 mg [^{14}C]N-nitrosodiethanolamine/kg body weight*

Tissue	No. of rats...	^{14}C activity (% of dose) after 4 hr	8 hr	24 hr	48 hr	96 hr	168 hr
		2	1	3	1	3	1
Stomach (+ contents)		0·05	0·01	0·04	0·04	0·03	0·05
Small intestine (+ contents)		0·05	0·05	0·07	0·06	0·09	0·24
Large intestine (+ contents)		0·03	0·02	0·07	0·05	0·11	0·09
Liver		0·05	0·05	0·04	0·10	0·17	0·3
Kidneys		0·07	0·04	<0·01	0·02	0·03	0·06
Brain		<0·01	<0·01	0·01	0·01	0·01	0·01
Adrenals		<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Pancreas		<0·01	0·01	<0·01	0·01	<0·01	0·01
Lungs		<0·01	0·01	0·01	0·01	0·01	0·02
Salivary glands		<0·01	<0·01	<0·01	<0·01	<0·01	0·01
Heart		<0·01	<0·01	<0·01	0·01	<0·01	0·01
Spleen		<0·01	<0·01	<0·01	<0·01	0·01	<0·01
Urinary bladder		<0·01	0·03	<0·01	<0·01	0·01	<0·01
Eye		<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Testes		<0·01	0·01	0·01	0·01	0·02	0·02
Thymus		<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Thyroid		<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Pituitary		<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Carcass		1·5	1·0	3·9	3·8	14·5	NT
Faeces		NT	NT	0·07	0·2	3·0	7·7
Urine		0·4	1·0	2·4	6·8	22·1	55·1
Bile		0·02	0·04	NT	NT	NT	NT
Expired CO_2		NT	NT	0·2	0·1	1·2	0·7
Skin, area of application		106·5	83·0	67·2	74·4	59·7	16·3

NT = Not tested

Values are means for the number of rats indicated.

Table 4. *The distribution of ^{14}C activity in male rats following a single topical dose of 50 mg [^{14}C]N-nitrosodiethanolamine/kg body weight*

Tissue	No. of rats...	^{14}C activity (% of dose) after 4 hr	8 hr	24 hr	48 hr	96 hr	168 hr
		2	1	1	1	3	1
Stomach (+ contents)		0	0	0·04	0·04	0·05	0·02
Small intestine (+ contents)		0·04	<0·01	0·07	0·07	0·1	0·05
Large intestine (+ contents)		<0·01	0	0·05	0·2	0·2	0·05
Liver		<0·01	0	0·08	0·1	0·2	0·2
Kidneys		0·02	0·1	0·01	0·02	0·6	0·03
Brain		<0·01	0	<0·01	0·01	<0·01	0·01
Adrenals		<0·01	0	<0·01	<0·01	<0·01	<0·01
Pancreas		<0·01	0	0·01	<0·01	0·01	<0·01
Lungs		0	0	0·01	0·01	0·01	0·02
Salivary glands		<0·01	0	<0·01	<0·01	<0·01	0·01
Heart		<0·01	0	<0·01	0·01	<0·01	<0·01
Spleen		<0·01	0	<0·01	<0·01	0·01	<0·01
Urinary bladder		<0·01	<0·01	0·06	<0·01	0·01	<0·01
Eye		<0·01	<0·01	0·01	<0·01	<0·01	0·1
Testes		<0·01	0	0·01	0·02	0·01	0·02
Thymus		<0·01	0	<0·01	<0·01	<0·01	<0·01
Thyroid		<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Pituitary		<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
Carcass		0·6	0·1	7·8	15·5	16·2	9·0
Faeces		NT	NT	0·7	0·7	2·0	7·7
Urine		0·2	0·2	3·0	15·2	19·3	46·3
Bile		<0·01	0·01	NT	NT	NT	NT
Expired CO_2		NT	NT	0·05	0·2	0·7	1·4
Skin, area of application		85·3	85·6	61·1	46·2	41·9	11·9

NT = Not tested

Values are means for the number of rats indicated.

About 8·0% of the dose was eliminated in the faeces. Less than 1·5% of ^{14}C was excreted as $^{14}CO_2$. Some ^{14}C was found in the bile at both dose levels at 4 and 8 hr. After 1 wk 12% of the high dose and 16% of the low dose remained on the skin at the site of application.

Column chromatography of urine and bile

Figure 1 shows representative Sephadex G-10 column scans of urine and bile samples after oral and topical administration of 0·5 mg NDELA/kg at 4 hr. The metabolic patterns were identical although the quantities varied. The major material in all the urine and bile samples was unmetabolized NDELA. A smaller unidentified material was eluted before the unchanged NDELA. Quantitative data obtained from the column separations of urine and bile are given in Table 5. No other metabolite was detected in any of the samples analysed.

DNA study

Results of the column chromatographic study of hydrolysed liver DNA indicated the absence of any alkylated purines. No measurable ^{14}C was detected in any of the column fractions.

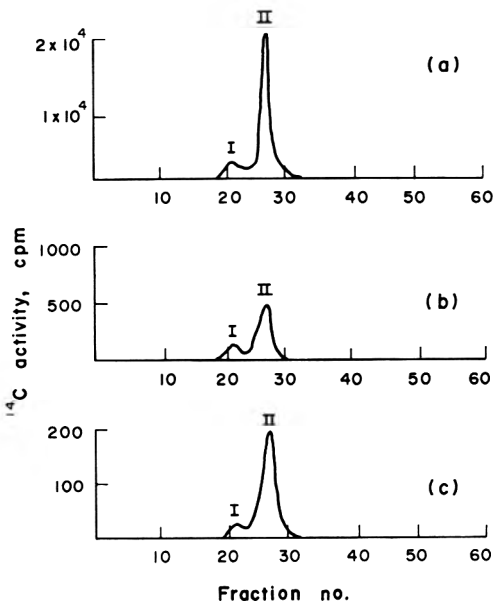

Fig. 1. Representative scans of fractions from Sephadex G-10 column chromatography of (a) urine and (b) bile samples from a male rat 4 hr after oral administration of 0·5 mg *N*-nitrosodiethanolamine/kg body weight, and (c) urine from a male rat 4 hr after dermal administration of 0·5 mg *N*-nitrosodiethanolamine/kg body weight. Peak I is the unidentified metabolite, peak II is NDELA.

DISCUSSION

After oral administration of [^{14}C]NDELA at levels of 0·5 and 50 mg/kg to male rats, the compound was rapidly absorbed from the gastro-intestinal tracts and distributed to all organs and tissues analysed. Tissue levels of ^{14}C reached a peak at 8 hr. After 1 wk the livers contained the highest levels of radioactivity, and some ^{14}C was found in all the organs. There was little difference in the distribution pattern due to the dose level.

After topical application of [^{14}C]NDELA at 0·5 and 50 mg/kg, the compound was slowly but continuously absorbed percutaneously over 1 wk. Radioactivity was found in the tissues within 4 hr after the application of NDELA, and substantial levels were found 1 wk after its administration. A considerable amount (12 and 16%, at the high and low doses, respectively) remained on the skin at the site of application. At the 50-mg/kg dose a significant level of ^{14}C was found in the eyeballs after 1 wk.

The major route of elimination by both orally and topically dosed rats was the urine. Only 3–8% of the

Table 5. *Composition of urinary and biliary radioactivity after administration of* [^{14}C]*N-nitrosodiethanolamine* ([^{14}C]*NDELA*) *to rats*

Route of administration	Dose (mg/kg)	Duration of experiment (hr)	Sex	Percentage of [^{14}C]NDELA present as	
				Metabolite	Unchanged NDELA
			Urine		
Oral	0·5	4	M	3·1	24·7
		8	F	5·3	53·1
		24	F	11·5	81·8
		24	M	9·1	73·2
	50·0	8	M	2·0	32·7
		24	M	11·3	76·8
Dermal	0·5	4	M	0·03	0·3
		8	M	0·08	0·9
		8	F	0·05	1·0
		24	M	0·3	2·1
	50·0	8	M	0·01	0·2
		24	M	0·3	2·7
			Bile		
Oral	0·5	4	M	0·25	0·65
Dermal	0·5	4	M	0·002	0·008
		8	M	0·01	0·02

dose was found in the faeces. Excretion of NDELA in the bile, although a minor route of elimination, may be significant since enterohepatic recycling could be the reason for the presence of substantial radioactivity in the animal body 1 wk after a single dose of NDELA. Biliary excretion would also explain the presence of ^{14}C in the intestinal tract of topically dosed rats 4–7 days after the application of NDELA to the skin. Also, the presence of ^{14}C in various tissues and organs 1 wk after the administration of a fairly rapidly excreted compound might reflect incorporation of labelled fragments into normal constituents of the rats, since only about 2% of the dose was eliminated as $^{14}CO_2$.

The amounts of NDELA found in the urine (3% or less at both dose levels) 24 hr after percutaneous absorption are not in agreement with the studies of Lijinsky *et al.* (1981), who found 20–30% of an undiluted 50-mg dose excreted in the urine after 24 hr. Lijinsky *et al.* (1981) reported the absorption of NDELA as almost complete within 1 hr, but in the present study 67% of the low dose and 61% of the high dose remained on the skin 24 hr after application. The difference in the absorption is probably not due to the dilution of NDELA with a small volume (less than 0·1 ml) of acetone in our study since the acetone would evaporate in a few seconds. However, the area of application could account for some of the difference; Lijinsky *et al.* used an area of application of 12·3 cm^2 compared with 4·9 cm^2 in our study.

Column chromatographic analyses indicated the same two ^{14}C components in the urine and bile samples from the orally and topically dosed rats. The major constituent was unmetabolized NDELA, and the other was a metabolite of NDELA. Once absorbed either orally or percutaneously, NDELA appears to be metabolized in the same manner regardless of dose level, sex, or time interval after dosing.

The induction of cancers by a number of *N*-nitrosamines is believed to be due to the formation of carbonium ion which in turn alkylates components of cellular tissue; specifically, alkylation has been detected in the guanine residues of DNA (Swann & Magee, 1971). The structure of NDELA differs from that of other carcinogenic nitrosamines in that it has a hydroxyl group attached directly to each ethyl group and therefore is not likely to be metabolized exactly like other nitrosamines.

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Fd Chem. Toxic. Vol. 20, pp. 407 to 412, 1982
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0278-6915/82/040407-06$03.00/0

EFFECT OF CYCLOPROPENOID FATTY ACIDS ON THE HEPATIC MICROSOMAL MIXED-FUNCTION-OXIDASE SYSTEM AND AFLATOXIN METABOLISM IN RABBITS

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(*Received 15 December 1981*)

Abstract—Male New Zealand weanling rabbits were fed a diet containing 0·25% cyclopropenoid fatty acids for 28 days. Compared with the controls, the rabbits given cyclopropenoid fatty acids showed retarded growth, some moderate liver histological damage, altered hepatic mixed-function-oxidase activities and minor variations in *in vitro* [^{14}C]aflatoxin B_1 metabolism. In *in vitro* assays the major hepatic metabolite of aflatoxin B_1 (AFB_1) was aflatoxicol (AFL) and the major AFL metabolite was AFB_1. Minor amounts of aflatoxin M_1 and a metabolite believed to be AFL-M_1 were formed. The similarity of this AFB_1 metabolite pattern to that in rainbow trout, taken together with the apparent absence of AFB_1 detoxification products is consistent with the sensitivity of both species to the acute effects of AFB_1.

INTRODUCTION

Dietary cyclopropenoid fatty acids (CPFAs) are responsible for a variety of biological effects in several animal species (Phelps, Shenstone, Kemmerer & Evans, 1965) including retarded growth in rats and chicks, delayed sexual development in female rats, and altered fatty acid metabolism and membrane function in rats (Nixon, Eisele, Wales & Sinnhuber, 1974). More recently, dietary CPFAs were demonstrated to be hepatocarcinogenic in rainbow trout (Sinnhuber, Hendricks, Putnam *et al.* 1976). CPFAs fed together with aflatoxin B_1 (AFB_1), a potent hepatocarcinogen in rainbow trout (Ayres, Lee, Wales & Sinnhuber 1971) and other animals (Campbell & Hayes, 1976) elicit a much higher tumour incidence in rainbow trout liver than the combined incidence resulting from exposure to each of the carcinogens alone (Lee, Wales, Ayres & Sinnhuber, 1968).

The liver microsomal mixed-function-oxidase system (MFO) biotransforms AFB_1 to initiate a carcinogenic response (Garner, 1973; Swenson, Lin, Miller & Miller, 1977). Studies in our laboratory show that dietary CPFAs alter the trout MFO system (Eisele, Nixon, Pawlowski & Sinnhuber, 1978) and alter the *in vitro* metabolism of AFB_1 (Loveland, Nixon, Pawlowski *et al.* 1979). CPFAs markedly enhance the hepatocarcinogenicity of AFB_1 (Lee, Wales & Sinnhuber, 1971), aflatoxicol (AFL; Schoenhard, Hendricks, Nixon *et al.* 1981), aflatoxin M_1 (AFM_1; Sinnhuber, Lee, Wales *et al.* 1974) and aflatoxin Q_1 (AFQ_1; Hendricks, Sinnhuber, Nixon *et al.* 1980).

The *in vitro* metabolism of AFB_1 is similar in liver preparations from rabbits and rainbow trout (Salhab & Edwards, 1977) and these two species are among the most sensitive to acute AFB_1 toxicity (Wogan, 1966). Rabbits (Ferguson, Wales, Sinnhuber & Lee, 1976) and trout also respond similarly to dietary CPFAs. Because of the similarities between rainbow trout and rabbits in their response to dietary CPFAs, AFB_1 metabolism and AFB_1 toxicity, the objective of this study was to determine whether dietary CPFAs alter the rabbit MFO system and *in vitro* metabolism of AFB_1.

This study was divided into two parts: identification of the AFB_1 metabolites formed *in vitro* by liver from control and CPFA-fed rabbits, and study of the effect of dietary CPFAs on rabbit growth, MFO system, and *in vitro* metabolism of [^{14}C]AFB_1.

EXPERIMENTAL

Animals and diets. Fourteen male weanling New Zealand rabbits were housed in individual cages at the Oregon State Laboratory Animal Resource Center and fed a pellet diet (OSU rabbit chow) consisting of 12·0% soya-bean meal, 10·0% alfalfa meal, 30·0% Pacific Coast barley, 27·3% corn, 5·0% fish meal, 10·0% whey, 2·0% feather meal, 2·5% bentonite, 0·5% salt, 0·7% Tricolphos, vitamin A (500,000 U/kg) and vitamin D (18,000 U/kg). After several weeks of feeding the control diet, five rabbits were selected at random and transferred to the experimental pellet diet containing the same ingredients plus 0·5% *Sterculia foetida* oil (SFO) for 28 days. The cyclopropene content of the SFO was 50·0% (Hammonds, Cornelius & Tan, 1971) giving a dietary concentration of 0·25% CPFA. Daily body weight and feed consumption were recorded on five control and five rabbits treated with SFO. These ten animals were used to study the effect of dietary CPFAs on the MFO system and the *in vitro*

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Abbreviations: AFB_1 = Aflatoxin B_1; AFB_{2a} = aflatoxin B_{2a}; AFL = aflatoxicol; AFM_1 = aflatoxin M_1; AFP_1 = aflatoxin P_1; AFQ_1 = aflatoxin Q_1; CPFA = cyclopropenoid fatty acid; MFO = mixed function oxidase; PMF = post-mitochondrial fraction; SFO = *Sterculia foetida* oil.

metabolism of [^{14}C]AFB_1 (see below). Two rabbits fed a control diet and two fed the CPFA diet for 28 days were used to study AFB_1 metabolite profiles.

Tissue preparation. Animals were fasted for 24 hr before they were killed. Body and organ weights were recorded, and tissue was taken for histology, CPFA analysis, and liver subcellular preparations. Livers were perfused with cold 0·9% NaCl, minced, homogenized in 4 vol (1:4, w/v) 0·15 M-KCl–0·05 M-potassium phosphate buffer, pH 7·4, and centrifuged at 2000 **g** for 15 min. The resulting supernatant was centrifuged at 10,000 **g** for 30 min to obtain a supernatant post-mitochondrial fraction (PMF). Microsomes and post-microsomal supernatant (supernatant) were obtained by centrifuging the PMF at 105,000 **g** for 60 min. The microsomal pellet was suspended in 0·05 M-potassium phosphate buffer (pH 7·4) such that 4 ml contained the equivalent of 1 g liver.

Histopathology. Tissues taken at autopsy were fixed in Bouin's solution. The liver, heart, coronary artery and aorta were embedded in paraffin. Sections (6 μm) were stained in haematoxylin and eosin.

Lipid extractions. Lipid was extracted from tissue by the procedure of Folch, Lees & Sloane Stanley (1957). Liver lipids were separated by silicic acid column chromatography into neutral and polar lipids (Roehm, Lee, Wales *et al.* 1970). Neutral lipids were eluted with 100 ml chloroform and the polar lipids, which contain primarily phospholipids, were eluted with 100 ml methanol. The CPFA content of the various lipid fractions was determined by the Halphen reaction as described by Hammonds *et al.* (1971).

In vitro *assays of AFB_1 and AFL metabolism.* The assay mixtures consisted of 1·00 ml 0·15 M-KCl–0·05 M-potassium phosphate buffer (pH 7·4), 0·95 ml of a buffered NADPH-generating system (containing 38 mg NADP, 320 mg glucose-6-phosphate and 40 mg $MgCl_2 . 6H_2O$/100 ml), and 0·02 ml (4 U) glucose-6-phosphate dehydrogenase. After 5 min preincubation at 37°C, 35 μl AFB_1 or AFL in methanol (1·128 mM) was added, followed by 2·00 ml of the appropriate liver fraction, PMF, post-microsomal supernatant or microsomes to bring the final volume to 4·00 ml.

Metabolite formation was found to be linear over the lower half of the range 0·5–5·5 mg protein/ml incubation mixture and for at least 30 min incubation time. For comparison studies between the two dietary groups, the parameters chosen were considered to be a compromise between linearity and having sufficient product to measure accurately. Therefore, protein concentrations for all liver fractions were adjusted to 2·5 mg protein/ml incubation mix, and the incubation time was 30 min.

The reactions were stopped by addition of an equal volume of acetone–chloroform (3:2, v/v). The remaining aqueous phase was extracted twice more with an equal volume (4 ml) chloroform. Organic extracts were combined, filtered through anhydrous Na_2SO_4, reduced to near dryness, and made up to 100 ml with CH_2Cl_2.

The metabolites were separated by TLC (silica gel 60 plates, E.M. Laboratories, Elmsford, NY) using as a solvent system either chloroform–acetone–isopropanol (82·5:15:2·2, by vol.) or benzene–acetone–ethyl acetate (55:15:30, by vol.). When checking for aflatoxin P_1 (AFP_1), several drops of glacial acetic acid were added to the solvent system. Known samples of AFB_1, AFL, AFM_1, AFB_{2a} and AFP_1 were used for TLC standards. Resolved spots were visualized by UV fluorescence. AFL-M_1 was confirmed by mass spectral analysis and TLC (Salhab & Edwards, 1977).

[^{14}C]*AFB_1 experiment.* The standard *in vitro* AFB_1 incubation (see above), with the addition of 0·4 μCi [^{14}C]AFB_1, was carried out using the PMF liver fraction. After the acetone–chloroform extraction, the aqueous fraction was re-extracted with 4 ml chloroform and centrifuged at 10,000 **g** for 15 min. The resulting aqueous phase was acidified with 10 ml 5% perchloric acid and centrifuged at 10,000 **g** for 15 min. The protein pellet was dissolved in 5 ml 5% NaOH. Aliquots of the various extracts were counted for radioactivity in aquasol or in toluene containing a PPO/POPOP fluor. Total radioactivity from the CH_2Cl_2 and chloroform extracts was termed the organic phase.

Metabolites from the CH_2Cl_2 extract were also separated by TLC using benzene–acetone–ethyl acetate (55:15:30, by vol.) as the solvent system. Spots corresponding to AFL and AFB_1 were scraped and radioactivity was determined by liquid scintillation counting in Aquasol.

Spectral studies and biochemical assays. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Assays for cytochrome *P*-450 and cytochrome b_5 content and the activity of NADPH–cytochrome *c* reductase were performed as described by LaDu, Mandel & Way (1971). Ethyl isocyanide (Sigma, St. Louis, MO) difference spectra (A_{455nm}/A_{429nm}) was measured at pH 7·4 in microsomes reduced with sodium dithionite according to the method of Norred & Wade (1972). The MFO system was characterized by measuring the activities of the following enzymes at 37°C: benzo[*a*]pyrene hydroxylase (DePierre, Moron, Johannesen & Ernster, 1975), ethoxyresorufin-*O*-deethylase (Burke, Prough & Mayer, 1977), ethoxycoumarin-*O*-deethylase (Ullrich & Weber, 1972), epoxide hydrase (James, Fouts & Bend, 1976), and glutathione transferase (James *et al.* 1976). The levels of serum cholesterol (Zlatkis & Zak, 1969) and reduced glutathione in liver (Hissin & Hilf, 1976) were also measured.

RESULTS

Growth

Rabbits that had been acclimatized to the control diet and then fed the diet containing CPFAs showed an initial sharp decline in growth followed by continual retarded growth compared to rabbits kept on the control diet (Fig. 1). The average amount of feed consumed/rabbit/day was 28% lower in the CPFA group (120 g) than in the control group (167 g). The average amount of feed required to produce 1 g of weight gain/day for the 28-day feeding period was 7·3 g for the controls and 13·7 for CPFA-fed rabbits, which represented an 88% decrease in feed conversion efficiency in the CPFA-treated animals.

Histopathology

Liver, heart, coronary artery and aorta from seven rabbits fed the diet containing CPFAs (five given

Fig. 1. Growth of rabbits given a control diet (○) or a diet containing cyclopropenoid fatty acids (□) for 28 days. The arrow indicates the start of feeding the test diet.

OSU rabbit pellets containing CPFAs and two from a preliminary experiment in which they were fed a semi-purified diet containing CPFAs for 28 days) were examined for histological changes. All of the rabbits had some fatty change within the liver, varying in degree from slight and diffuse in four rabbits to moderate and more centrilobular in the other three. In addition, the livers from two rabbits had congested sinusoids and foci of hepatocytes undergoing severe hydropic degeneration. Moderate amounts of diffusely distributed ceroid pigment, some necrosis, and infiltration of polymorphonuclear leucocytes were associated with the degenerative foci in one of these livers. Livers from three rabbits fed a CPFA-free diet were examined and did not show any of the microscopic changes described.

Except for an excessive amount of adipose tissue in the epicardium of the heart from one CPFA-fed rabbit, no other tissue examined appeared to be affected.

CPFA content of tissue lipids

The adipose lipids contained an average of 14·7% CPFA, whereas the kidney and liver lipids contained 1·1 and 2·8% CPFA, respectively. Liver lipid was fractionated into a neutral and polar (phospholipids) fraction and each fraction contained 0·5% CPFA. The results indicate that dietary CPFAs were deposited within the lipids of the major tissues and organs as well as in the phospholipids of the liver.

In vitro *assays of AFB_1 and AFL metabolism*

The major *in vitro* metabolite of AFB_1 was AFL, which was produced by the PMF and supernatant fractions (Table 1). The microsomal fraction produced a small amount of AFM_1 and a trace of AFL, which probably arose from some contamination by the supernatant fraction. The major *in vitro* metabolite of AFL was AFB_1, which was formed by the PMF and supernatant fractions. Both the reductive enzyme for the conversion of AFB_1 to AFL and the dehydrogenase enzyme for the conversion of AFL to AFB_1 were located primarily in the 105,000-**g** supernatant. Salhab & Edwards (1977) were able to separate the two enzymes in a number of animal species by centrifuging the PMF fraction at 225,000 **g**, resulting in a sedimented enzyme acting as an AFL to AFB_1 dehydrogenase. Loveland *et al.* (1979) were able to partially sediment the rainbow trout dehydrogenase enzyme at 105,000 **g**. We were unable to separate the two rabbit enzymes at 105,000 **g**.

A metabolite formed in trace amounts, AFL-M_1 (a derivative of AFM_1 in which the cyclopentenone was reduced to the alcohol equivalent in AFL), was formed from AFB_1 by the PMF, but not by the microsomes or the supernatant fraction, and from AFL by the PMF or microsomes, but not by the supernatant. Spots which co-chromatographed with AFQ_1 and AFB_{2a} were barely visible in the TLC chromatogram of the AFB_1 incubations with the microsomal fraction. Two fairly prominent fluorescent spots in the same microsomal AFB_1 incubation extract were unidentified. AFP_1 was absent from all TLC chromatograms.

The metabolite profile for both AFB_1 and AFL of CPFA-fed rabbits was quantitatively similar to the controls. The only substantial difference seemed to be a slightly larger conversion of AFL to AFB_1 by the supernatant fraction from CPFA-fed rabbits.

Comparison of in vitro *[^{14}C]AFB_1 metabolism of CPFA-fed rabbits with controls*

In view of the fact that AFL was the major metabolite formed from AFB_1, the effects of dietary CPFAs on the *in vitro* conversion of AFB_1 to AFL were

Table 1. *Metabolites of AFB_1 and AFL in liver subcellular preparations from control rabbits*

Substrate	Liver fraction*	Metabolites identified: Major	Trace†	Tentative†
AFB_1	PMF	AFL	AFM_1, AFL-M_1	AFB_{2a}
	Supernatant	AFL		
	Microsomes		AFM_1, AFL	AFQ_1, AFB_{2a}
AFL	PMF	AFB_1	AFL-M_1	
	Supernatant	AFB_1		
	Microsomes		AFB_1, AFL-M_1	

*The liver fractions were as follows: PMF (post-mitochondrial fraction) = 10,000-**g** supernatant; supernatant = 105,000-**g** (post-microsomal) supernatant; microsomes = 105,000-**g** pellet.

†Trace = very small amounts, usually <2% substrate; tentative = barely visible on TLC chromatogram by UV fluorescence.

examined next, using [^{14}C]AFB_1. The results (Table 2) indicate that 28 days of administration of CPFAs in the diet had a minor effect on the metabolism of AFB_1. There was less conversion of AFB_1 to AFL in CPFA-fed rabbits compared with the controls, thus causing an increase in the AFB_1 pool. Also, less AFB_1 was bound to microsomal protein (whether expressed as percentage radioactivity or mg AFB_1 bound g protein) in CPFA-fed rabbits. Although the effect of dietary CPFAs on the *in vitro* metabolism of AFB_1 by rabbit-liver preparations was not statistically significant ($P > 0.05$) and was less dramatic than that observed for CPFA-fed rainbow trout (Loveland *et al.* 1979), the trend, when expressed as a percentage difference, suggested less conversion of AFB_1 to AFL and less AFB_1 binding to microsomal protein.

Comparison of MFO levels and activities

The effect of 28 days of administration of CPFAs in the diet on the rabbit MFO system is shown in Table 3. In general, dietary CPFAs caused a decrease in MFO enzyme activities as well as in cytochrome *P*-450 and b_5 content. The *O*-deethylase enzymes and benzo[*a*]pyrene hydroxylase activities were reduced to a greater degree by dietary CPFAs while NADPH–cytochrome *c* reductase activity was slightly elevated. There was no statistically significant difference ($P > 0.05$) between the two dietary groups. However,

Table 2. [^{14}C]AFB_1 *conversion to AFL and water-soluble metabolites and binding to microsomal protein in post-mitochondrial liver fractions from rabbits fed diets containing CPFAs or control diets*

Parameter	Extract*	Values† for rabbits given: Control diet	Values† for rabbits given: CPFA diet	Difference between values (% of control)
^{14}C (% of dose) present in extracts	Organic: total	81·90 ± 3·48	79·68 ± 4·14	−2·7
	Organic: as AFB_1	47·60 ± 5·46	49·00 ± 6·96	+2·9
	Organic: as AFL	22·20 ± 4·82	19·00 ± 5·79	−14·4
	Aqueous	5·33 ± 0·64	5·33 ± 0·53	0
	Protein	2·55 ± 0·22	2·36 ± 0·43	−7·5
AFB_1 bound to microsomal protein (mg g protein)	—	30·64 ± 3·25	27·96 ± 6·30	−8·7

CPFAs = Cyclopropenoid fatty acids AFB_1 = Aflatoxin B_1 AFL = Aflatoxicol

*The incubation mixtures were extracted with acetone–chloroform (3:2, v v) and with chloroform and the organic extract was developed by thin-layer chromatography. The aqueous phase was acidified and centrifuged to obtain the protein extract. For further details see Experimental.

†Values are means ± 1SD for groups of five rabbits.

Table 3. *Effect of dietary CPFAs on the hepatic mixed-function-oxidase system and levels of hepatic reduced glutathione and serum cholesterol in rabbits*

Parameter	Values* for rabbits given: Control diet	Values* for rabbits given: CPFA diet	Difference between values (% of control)
Relative liver weight (% of body weight)	3·38 ± 0·40	3·58 ± 0·32	+6
Microsomal protein (mg g liver)	6·79 ± 2·10	7·14 ± 2·31	+5
Cytochrome *P*-450 (nmol mg microsomal protein)	0·702 ± 0·135	0·513 ± 0·177	−27
Cytochrome b_5 (nmol mg microsomal protein)	0·285 ± 0·111	0·158 ± 0·135	−45
NADPH–cytochrome *c* reductase†	36·66 ± 9·04	40·34 ± 13·34	+10
Benzo[*a*]pyrene hydroxylase†	0·190 ± 0·109	0·114 ± 0·118	−40
Ethoxyresorufin-*O*-deethylase†	0·12 ± 0·08	0·08 ± 0·05	−33
Ethoxycoumarin-*O*-deethylase†	0·29 ± 0·13	0·25 ± 0·13	−14
Epoxide hydrolase†	4·70 ± 1·92	4·72 ± 3·58	0
Glutathione transferase‡	40·39 ± 5·39	35·92 ± 9·25	−11
Ethylisocyanide difference spectrum (A_{455nm} A_{429nm})	0·13 ± 0·05	0·13 ± 0·04	0
Hepatic reduced glutathione (μg g liver)	1068 ± 206	1067 ± 156	0
Serum cholesterol (mg 100 ml serum)	131·7 ± 15·6	184·7 ± 111·0	+40

CPFAs = Cyclopropenoid fatty acids

*Values are means ± 1 SD for groups of five rabbits.

†Determined in microsomal fractions (see Experimental) and expressed as nmol mg microsomal protein min.

‡Determined in the post-microsomal (105,000 **g**) supernatant and expressed as nmol mg post-microsomal supernatant min.

expressing the data (Table 3) as a percentage difference between the groups suggests that dietary CPFAs did affect the rabbit MFO system in a manner similar to that seen in the rainbow trout MFO system (Eisele *et al.* 1978).

The ethylisocyanide difference spectrum of sodium-dithionite-reduced microsomes indicated no difference between dietary groups, suggesting that the acute dietary doses of CPFAs did not alter cytochrome *P*-450 in rabbits. Total liver reduced glutathione levels were also unaffected by dietary CPFAs (Table 3). Serum cholesterol levels were found to be elevated by 40% in CPFA-fed rabbits compared to controls; a similar observation was made by Ferguson *et al.* (1976).

DISCUSSION

The effects of 28 days of feeding CPFAs at 0·25% in the diet on several physiological parameters, the MFO system, and on *in vitro* hepatic AFB_1 metabolism were examined in the rabbit. CPFA-fed rabbits consumed 28% less feed than controls and exhibited an 88% decrease in feed conversion. Reduced food consumption obviously contributed to the retarded growth, but low feed conversion efficiency was also a major factor responsible for the poor growth of CPFA-fed rabbits. This same observation has been reported by Nixon *et al.* (1974) with rats fed 1% CPFA. Since CPFAs are found at levels of 0·56–1·17% in cottonseed oil (Bailey *et al.* 1966) and can cause reduced growth and poor feed conversion efficiency, the use of cottonseed oil in commercial rabbit rations should be reconsidered.

The dietary CPFAs were readily incorporated into the lipids of adipose and liver tissue with a substantial amount found in liver phospholipids (0·5%). This is of particular importance because cellular membranes are composed of phospholipids and altering membrane lipids, as noted by several authors (Decker & Mertz, 1967; De Kruyff, Van Dijck, Goldbach *et al.* 1973; Nixon *et al.* 1974), alters the physical and biochemical geometry of membranes. Thus, the inclusion of ring structured fatty acids, such as CPFAs, could also alter membrane molecular geometry.

A number of *in vivo* experiments have shown that chemical carcinogenesis can be inhibited or induced by altering MFO enzyme activities (Clayson, 1975; Wattenberg, 1974 & 1975). This report indicates that dietary CPFAs may alter MFO enzyme activity in rabbits (Table 3). The *in vitro* metabolism of $[^{14}C]AFB_1$ by CPFA-fed rabbits was slightly altered compared to controls (Table 2). CPFA-fed rabbits tended to convert less AFB_1 to AFL, and AFB_1 binding to protein was lower. The data suggest that the CPFA-altered MFO system might be responsible for the slight change in *in vitro* AFB_1 metabolism. The mechanism by which CPFAs interfere with AFB_1 binding to protein or alter the conversion of AFB_1 to AFL in rainbow trout or in rabbits is unknown. However, the MFO system is known to have a requirement for lipid (Marshall & McLean, 1971) and resides on the lipid-rich membrane of the endoplasmic reticulum. It is possible that CPFAs, through the alteration of membrane geometry, could interfere with the detoxifying mechanism of AFB_1 rather than interfering with the activation of the carcinogen. Such a process would cause a larger AFB_1 pool in CPFA-fed animals resulting in more of the 'ultimate carcinogen' or epoxide.

The ability of rabbit-liver enzymes to convert AFB_1 to AFL and back to AFB_1 with little conversion to oxidative detoxification products may explain why rabbits were found to be almost as acutely sensitive to AFB_1 as rainbow trout (Wogan, 1966). The apparent carcinogenicity of AFL (Nixon, Hendricks, Pawlowski *et al.* 1981; Schoenhard *et al.* 1981) seems to involve more than the ability of AFL to be converted back to AFB_1. Since $AFL\text{-}M_1$ can be formed by the action of the MFO enzymes, there is a strong probability that AFL can be converted to an epoxide in a manner analogous to AFB_1.

In summary, in rabbit liver preparations *in vitro* the major metabolite of AFB_1 was AFL and the major metabolite of AFL was AFB_1. Rabbits fed CPFAs for 28 days exhibited retarded growth, had altered MFO enzymatic activities, and had some abnormal changes in liver histology. *In vitro* metabolism of $[^{14}C]AFB_1$ in CPFA-fed rabbits was similar to that in controls. The data demonstrate that rabbits metabolize AFB_1 and AFL, and respond to dietary CPFAs in a similar manner to, but not as pronounced as, the response in rainbow trout.

Acknowledgements—The authors thank J. S. Wilcox, J. L. Williams and D. W. Shelton for their technical assistance. This work, published as Technical Paper No. 6154, of the Oregon Agricultural Experiment Station, was supported in part by USPHS grants ES00550 and ES00541 and NCI grant CA25766.

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Fd Chem. Toxic. Vol. 20, pp. 413 to 416, 1982
Printed in Great Britain

0278-6915/82/040413-04$03.00/0
Pergamon Press Ltd

PROBLEMS ASSOCIATED WITH THE USE OF CYCLOHEXIMIDE TO DISTINGUISH BETWEEN ANIMAL DRUG RESIDUES BOUND TO PROTEIN AND THOSE INCORPORATED INTO PROTEIN

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(*Received 11 November 1981*)

Abstract—The use of cycloheximide to distinguish between covalently-bound drug residues in animals and residues due to the incorporation of drug fragments into endogenous molecules was explored. The results indicated that cycloheximide prevented the absorption of both glycine and ronidazole from the gastro-intestinal tract, an effect that complicates its use in the characterization of drug residues in animals.

INTRODUCTION

A consequence of the administration of drugs to food-producing animals is that residues of these drugs may be present in the tissues of the animals at the time of slaughter. Assessing the safety of these residues is sometimes complicated by the fact that their chemical nature is unknown. Furthermore, some types of residue are intractable in that they cannot be separated from the tissue macromolecules by the usual extraction or other separation procedures. Residues may be intractable because they are physically complexed with the macromolecules in a manner preventing liberation of the residue, metabolites can be covalently bound to the macromolecules, or the intractable residue may actually be radioactive fragments of the drug molecules incorporated into macromolecules by normal metabolic routes. This last category of drug residue, that due to incorporation into endogenous components, is of course the safest, since it is not drug related but is a drug residue by virtue of the fact that radioactivity is used to measure residue levels. It is probably the most difficult to identify and quantitate since the number of molecules into which the radioactivity is incorporated is extremely large. If the amount of drug residue that is not drug related could be quantitated, this fraction could be eliminated from the assessment of the safety of the residue, since it is not of toxicological concern.

One method proposed (Weber, 1980) for distinguishing between non-drug-related residues, which are drug fragments incorporated into normal endogenous compounds, and drug-related residues is the use of protein-synthesis inhibitors. This approach has some intrinsic problems. Protein synthesis cannot be inhibited to a great extent for long periods of time without severely affecting the animal and compromising its drug-metabolizing ability. The period free of such effects is probably less than 24 hr, but withdrawal periods for many animal drugs are of the order of days if not weeks. This would not be a problem if the animal drug had a very short half-life, so that the drug residue present at the time of slaughter was formed while protein synthesis was being inhibited. Since this is not usually the case, the residues that are examined at the chosen time-point may not represent the residues of concern at the time of slaughter.

Despite these drawbacks we undertook to determine, through the use of protein-synthesis inhibitors, whether the intractable drug residue from ronidazole, a 5-nitroimidazole developed for the treatment of swine dysentery and turkey blackhead, was due partially to fragments of the molecules that had been incorporated into proteins. Following metabolism in turkeys and rats, radioactivity from this compound was identified in oxalic acid, glutamic acid and methionine, indicating the formation of one- and two-carbon fragments (Rosenblum, Trenner, Buhs *et al.* 1972; authors' unpublished data 1975). Another possible fragmentation product could be glycine. Therefore we examined the effect of cycloheximide, a classical protein-synthesis inhibitor (Gorski & Axman, 1964), on the levels of radioactivity in various rat tissues and in TCA precipitates of these tissues. Glycine was used as a positive control. Problems encountered in these studies are the subject of this paper.

EXPERIMENTAL

Chemicals. [^{14}C]Ronidazole was synthesized by the Radiochemistry group at Merck. The compound was labelled in the 4 and 5 positions of the imidazole ring. The specific activity of the ronidazole ranged from 5 to 15 μCi/mg. The [^{14}C]glycine was obtained from Research Products International, Elk Grove Village, IL. The specific activity of the glycine used ranged from 400 to 500 μCi/mg. Cycloheximide and glycine were obtained from Sigma Chemical Co., St. Louis, MO.

Animals. Sprague–Dawley male rats, 8–12 wk of age, were used in the experiments. The rats were given cycloheximide ip in a dose of 2 mg/kg as a 2-mg/ml solution in saline, 1 hr before the glycine or ronida-

Abbreviation: TCA = Trichloroacetic acid.

zole treatment and 4·5 hr after. Ronidazole was dosed at 10 mg/kg in saline (2 mg/ml) while glycine in a concentration of 0·113 mg/ml saline was administered in a dose of 0·375 mg/kg in the first experiment and 0·25 mg/kg in the second experiment. These compounds were dosed either by gavage or by iv injection into the femoral vein. The rats were housed in metabolism cages that permitted the separate collection of urine and faeces. Blood samples were taken through the orbital sinuses with the rats under light ether anaesthesia or by heart puncture at the end of the experiment. The rats were killed by cervical dislocation 7·5 hr after the administration of the glycine or ronidazole and the tissues of interest were removed.

Analysis for radioactivity. The tissues were homogenized in 3 vols water using a Virtis "45" Hi Speed Homogenizer. A sample of the homogenate was then analysed directly, by combustion, and the protein precipitated from the homogenate with TCA was also analysed. Direct analysis of the tissues involved combustion of 0·5-g samples of the homogenates in a Packard 306 sample oxidizer (Packard Instrument Co., Chicago, IL) and trapping of the $^{14}CO_2$ formed in a mixture of 8 ml Carbsorb (Packard) and 13 ml Permafluor V (Packard). The samples were then counted in a Packard scintillation counter. TCA precipitation of the protein was achieved by treating 1 ml of the homogenate with 2 ml 0·9 M-TCA. The resulting precipitate was collected by centrifugation and washed twice with 3 ml 0·6 M-TCA and twice with 3 ml 80% methanol. The samples were dissolved in Soluene-350 (Packard) and an aliquot was counted using Dimilume-30 (Packard) as the counting cocktail.

RESULTS

Since the field use of ronidazole involves its oral administration, the oral route was used also for the administration of ^{14}C-labelled glycine. As was expected, treatment of the [^{14}C]glycine-treated rats with cycloheximide lowered the amounts of radioactivity in their tissues (Table 1). The degree of inhibition was about 90% in the kidney, liver and muscle tissue. TCA precipitation of the protein of these fractions indicated approximately 95% reduction of radioactivity levels in the TCA precipitate compared to the control levels. Unexpectedly, the combustion analyses showed in fat a degree of inhibition similar to that in the other tissues, while in the carcass (body minus liver, kidneys and skin) radioactivity was higher than the control levels.

This experiment was repeated using [^{14}C]ronidazole instead of glycine. Again inhibition of ^{14}C incorporation was associated with cycloheximide treatment although it was not as great as that observed in the glycine experiments (Table 1). The level of inhibition was from 50 to 60% in liver, kidney and muscle tissue and in the 40–65% range in the TCA precipitates. The level of radioactivity in fat was reduced by nearly 60%. The carcass of the cycloheximide-treated animals again showed levels of radioactivity in both the whole homogenate and in the TCA-precipitable fraction greater than those in the control rats.

The differences between the control rats and those treated with cycloheximide could have been due either to the prevention of incorporation of radioactivity into protein by the cycloheximide or to prevention of absorption from the gastro-intestinal tract. Since the gut remains with the carcass, the higher level of radioactivity found in the carcasses of the cycloheximide-treated rats could be accounted for by a lower absorption rate. To examine this possibility, the stomach and intestines were removed from the carcasses and levels of radioactivity in these tissues as well as in the plasma of the ronidazole-treated rats were determined.

Following [^{14}C]ronidazole administration, the amount of radioactivity in the stomachs of the cycloheximide-treated rats was more than 2·5 times greater than that in the stomachs of the control rats. Consistent with this finding was the observation that the in-

Table 1. *Cycloheximide inhibition of incorporation of radioactivity from orally administered* [^{14}C]*glycine or* [^{14}C]*ronidazole into the tissues and TCA precipitates of the tissues of rats*

Sample	Treatment group	^{14}C activity (dpm/mg tissue*)				
		Liver	Muscle	Kidney	Fat	Carcass†
		[^{14}C]Glycine-treated rats				
Tissue homogenate	Control	676	180	453	73	284
	Cycloheximide	71 (10)	17 (9)	43 (9)	11 (15)	493 (174)
TCA precipitate	Control	493	75	281	—	91
	Cycloheximide	26 (5)	2·4 (3)	14 (5)	—	217 (238)
		[^{14}C]Ronidazole-treated rats				
Tissue homogenate	Control	38	29	57	12	102
	Cycloheximide	17 (45)	15 (52)	23 (40)	5·3 (44)	141 (139)
TCA precipitate	Control	6·8	2·1	4·0	—	20
	Cycloheximide	2·6 (38)	0·8 (36)	2·4 (60)	—	23 (113)

*Values for ^{14}C incorporation in rats treated with cycloheximide are also expressed (in brackets) as a percentage of the value for the corresponding control group given the labelled compound but no cycloheximide. Values are means of the results for three rats except those for the carcass, which in the case of the glycine-treated rats are each derived from one animal.

†Body minus liver, kidneys and skin.

Table 2. *Radioactivity in the tissues and TCA precipitates of the tissues of rats given [^{14}C]glycine by iv injection or by gavage with and without prior treatment with cycloheximide*

Treatment	[^{14}C] activity (% of control given glycine iv and no CH)							
	Liver	Muscle	Kidney	Fat	Stomach	UI	LI	Carcass*
Tissue homogenates†								
Glycine (iv) + CH	133	102	99	232	26·6	116	105	84
Glycine (oral) + CH	19·1	3·5	6·8	45·8	955	15·0	27·7	7·3
Glycine (oral), no CH	150	103	112	191	252	393	254	97
TCA precipitates§								
Glycine (iv) + CH	47·3	23·4	33·5	—		—		29·1
Glycine (oral) + CH	18·4	3·1	4·2	—		—		2·4
Glycine (oral), no CH‡	151	84·7	94	—		—		101

CH = Cycloheximide UI = Upper half of intestine LI = Lower half of intestine

*Body minus liver, kidney, skin, stomach and intestines.

†Values from analysis of the tissues of one rat in each group.

‡Oral control.

§Values are means of the results for three rats, except for the oral control group (two rats) and the carcass data (one rat only in each group).

itial plasma level of radioactivity in the control group was twice as high as that in the cycloheximide-treated rats.

Since ronidazole might be unique in its distribution in rats treated with cycloheximide, we examined in more detail the effect of cycloheximide on the amounts of radioactivity in the tissues of rats given [^{14}C]glycine either by iv injection or oral gavage (Table 2). The data are expressed using the levels of radioactivity in the tissues of the control rat given an iv dose of [^{14}C]glycine as 100%. In the rat given the iv injection of glycine after cycloheximide treatment, the tissue levels of radioactivity in the liver, muscle, kidney and carcass were not very different from the corresponding values for the iv control rat. However, the radioactivity level in fat was more than twice as high, while that in the stomach was only 27% of the control level. The levels of radioactivity in the tissues of rats given an oral dose of glycine and cycloheximide were very different from both the iv-injected control and the control given only glycine orally. Levels decreased by 60–97% were observed in all the tissues except the stomach, in which the level was nine times as high as that in the iv injected control. The rat given glycine orally without cycloheximide treatment had tissue levels of radioactivity generally higher than those of the iv control, especially in the gastro-intestinal tract. An examination of the relative amounts of radioactivity in the TCA precipitates of the orally-dosed control rat showed some similarities to the iv dosed control rats (Table 2). Only the TCA precipitate of the liver of the orally dosed rats showed a marked increase in the level of radioactivity. In rats given glycine orally with cycloheximide treatment, the levels of radioactivity in the TCA precipitate were reduced to 80–95% of the control levels. This was similar to the reduction seen in the homogenate of the tissues of these rats. Interestingly, in the rats treated with cycloheximide and given glycine iv, the levels of radioactivity were reduced by about 50–75%.

DISCUSSION

The results of the early experiments with glycine and ronidazole (Table 1) did not appear to be as straightforward as expected. The subsequent data indicated that cycloheximide can act in more than one way to decrease the amount of radioactivity in TCA precipitates of the tissues of rats that have been treated orally with [^{14}C]glycine. The higher levels of radioactivity in the stomach indicate that the lower levels of radioactivity in the liver, muscle, kidney and carcass are most probably due to the fact that cycloheximide inhibits the absorption of glycine from the gut. The reduced levels of radioactivity in the tissues of these rats are also probably caused by an inhibition of incorporation of glycine, as indicated in the data from the rats treated with glycine iv and cycloheximide (Table 2). The levels of radioactivity in the TCA precipitates of the tissues of these rats was also reduced but to a much smaller extent than following oral glycine treatment (Table 2). At least with cycloheximide therefore, the oral route of administration of the drug (ronidazole) is a poor choice. However, if this is the normal route of administration of a drug under study one is faced with a problem, since the route of administration could affect the distribution and metabolism of the drug. Results that might indicate that certain percentages of the intractable residues following iv administration were due to endogenous incorporation might not be applicable to other routes of administration.

A further complication of this technique is the choice of a positive control. To ascertain how effective a protein-synthesis inhibitor would be in inhibiting incorporation of metabolites of a drug into endogenous macromolecules, one would have to know what the metabolites of the drug were and the amounts formed. If this were known completely there might not be any reason to do the experiment. Clearly the type of metabolites, whether they be acetate, oxalate, formaldehyde, bicarbonate or CO_2, would have an influence on how effective the protein-synthesis inhibitor would be. Because of the problems discussed here and in the introduction, it is very difficult to assess quantitatively the contribution that the incorporation of radioactivity into endogenous compounds makes to the residue level. It would be relatively easy to determine that radioactivity is incorporated into certain endogenous substances through the use of the

reverse isotope dilution assay on tissues from animals treated with the drug under study. However, what is required to remove from toxicological concern residues that are not drug related in nature is a quantitation of these residues at the withdrawal time. Since the use of cycloheximide does not usually permit this quantitation, there are probably only a few instances in which it might be useful.

Acknowledgements—The authors would like to thank Miss Margaret Pastor for her assistance in dosing the animals by the iv route. We also thank Mrs Donna Gibson for her excellent assistance in the preparation of this manuscript.

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Fd Chem. Toxic. Vol. 20, pp. 417 to 425, 1982
Printed in Great Britain.

0278-6915/82/040417-09$03.00/0

COMPARATIVE RATES OF ELIMINATION OF SOME INDIVIDUAL POLYCHLORINATED BIPHENYLS FROM THE BLOOD OF PCB-POISONED PATIENTS IN TAIWAN

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(*Received 11 November 1981; revision received 11 January 1982*)

Abstract—In 1979, a mass outbreak of poisoning occurred in Central Taiwan due to the ingestion of rice-bran oil contaminated with polychlorinated biphenyls (PCBs). The major PCB isomers and congeners in the toxic rice oil and in the blood of PCB-poisoned patients were characterized by gas chromatography and gas chromatography mass spectrometry using a highly efficient glass capillary column. The elimination of some major individual PCBs from blood of these patients was studied. The results indicate that tetra- and pentachlorobiphenyls with adjacent unsubstituted carbon atoms at *meta–para* positions are rapidly eliminated from the blood of patients, while PCBs with the same degree of chlorination but with adjacent unsubstituted carbon atoms at *ortho–meta* positions are eliminated more slowly. The results also indicate that most of the hexa- and heptachlorobiphenyls, with adjacent unsubstituted carbon atoms at *ortho–meta* positions of the biphenyl ring, are eliminated very slowly. Laboratory-animal studies have indicated that PCB excretion depends primarily on the rate of metabolism; therefore these differences in rates of elimination of PCBs should reflect the differences in their rates of metabolism.

INTRODUCTION

The problems of polychlorinated biphenyls (PCBs) have aroused a great deal of concern because of their widespread occurrence and persistence in the environment and their tendency to accumulate in food chains, with possible adverse effects on human health (Wasserman, Wassermann, Cucos & Miller, 1979). The first most serious incident of PCB poisoning in man occurred in Western Japan in 1968 (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972), the total number of persons affected being estimated in 1980 as about 1700. In 1979, a similar mass outbreak of PCB poisoning in Taichung and Changhwa in Central Taiwan resulted from the ingestion of rice-bran oil contaminated with PCBs (Chen, Chang & Lu, 1981; Chen, Gaw, Wong & Chen, 1980). By the middle of 1981, the total number of reported cases in this incident was more than 2000.

It has been established that PCBs are readily absorbed from the gastro-intestinal tract and rapidly distributed by the blood to all tissues (Matthews & Anderson, 1975). After their absorption and distribution, the excretion of PCBs depends primarily on their rates of metabolism (Matthews & Anderson, 1975). Laboratory animal studies showed that the less chlorinated PCB congeners were more readily metabolized than the more highly chlorinated PCB components (Matthews & Anderson, 1975). The rate of PCB metabolism is also dependent on the position of the chlorine substituents (Matthews & Tuey, 1980).

In a previous publication (Chen *et al.* 1980), we reported the levels and GC patterns of PCBs in blood samples taken from patients between 9 months and 1 yr after they had been poisoned with PCBs. Since January 1980 we have analysed a large number of blood samples from PCB-poisoned patients. In addition to determining the total PCB level in the blood, we have been particularly interested in the change in the concentration of individual PCB isomers and congeners in the blood of a given patient at different times during the post-poisoning period. We have studied the changes in the GC pattern of PCBs in the blood of 17 patients, and this has enabled us to investigate the comparative ease of elimination of some PCB isomers and congeners from the blood of PCB-poisoned patients. This paper described the results of this study.

EXPERIMENTAL

Blood and oil samples. Blood samples of PCB-poisoned patients were collected in the hospital ward and the out-patient clinic of the Department of Dermatology, Veterans General Hospital, Taipei, and in patients' homes during the period January 1980–June 1981. The toxic rice-bran oil sample analysed for PCBs was one of several oil samples obtained from different sources in Taichung.

Reagents. Kanechlor 500 was a gift from Prof. Y. Masuda of Daiichi College of Pharmaceutical Sciences, Japan. 2,5,2′,4′,5′-Penta-, 2,4,5,2′,4′,5′-hexa-

Abbreviations: ECD = Electron capture detector; GC = gas chromatography; MS = mass spectrometry; PCB = polychlorinated biphenyl.

and 2,3,4,2',4',5'-hexachlorobiphenyls were purchased from Analabs, North Haven, CT, USA, for use as reference standards. Silica gel (Wakogel S-1) and anhydrous sodium sulphate were purchased from Wako Pure Chemical Industries, Osaka, Japan. *n*-Hexane and absolute alcohol were obtained from E. Merck AG, Dortmund, FRG.

Isolation and purification of PCBs. The method for the isolation of PCBs from the blood samples and from the toxic rice-oil sample was essentially the same as that used by us in our previously reported work (Chen *et al.* 1981 & 1980). This method includes the saponification of the whole blood or the rice oil with an alkali, followed by extraction of PCBs with *n*-hexane. The condensed extract was cleaned up by silica-gel column chromatography using *n*-hexane as the eluting solvent.

Gas-chromatographic analysis. The purified extract was analysed for PCBs by GC, using a Shimadzu GC-6AM equipped with a ^{63}Ni electron capture detector (ECD). The open tubular column used was a 30 m × 0·25 mm ID glass capillary column coated with SE-30 (Wako Pure Chemical Industries). The glass capillary column was installed in the GC with the aid of a Shimadzu CLH-4M micro-column holder. Column temperature was maintained at 200°C and carrier-gas (N_2) flow was about 0·6 ml/min. Nitrogen make-up gas was fed at 30 ml/min through the capillary column outlet tee.

Gas-chromatographic/mass-spectrometric analysis. The purified *n*-hexane extract of the toxic rice oil was also analysed for PCBs by a computer-controlled GC/MS (HP 5985A) operating in the selected ion monitoring mode (Falkner, Sweetman & Watson, 1975) to enhance the instrument's sensitivity. The purpose of this analysis was to determine the number of chlorine atoms in each PCB peak. The column used was a 30 m × 0·25 mm ID glass capillary column coated with SE-30. The column was operated at 200°C with a column helium flow rate of about 0·5 ml/min.

Retention indices. A flame ionization detector was necessary as *n*-alkanes (C_{18}, C_{20}, C_{22}, C_{24}) were added to Kanechlor 500 and the toxic rice-oil extract for the determination of retention indices (Sissons & Welti, 1971). The same open tubular column and conditions described above for the GC analysis were used for this experiment.

ECD response factors for individual PCBs. ECD response factors for some PCBs were determined by injecting a known amount of the authentic PCB reference standards into the open tubular column and comparing the relative area responses of the ECD. In cases where PCB reference standards were not available to us, the ECD response factors were determined by dividing the area responses of the ECD for the individual PCBs in Kanechlor 500 or the toxic rice oil by the weight percentage of the appropriate PCB in Kanechlor 500 or the toxic rice oil, as determined by high-resolution GC using a flame ionization detector.

Total PCB level in blood. The total PCB level in the blood of each patient was determined by the PCB quantification method described by Ugawa, Nakamura & Kashimoto (1973).

Table 1. *Comparative rates of elimination of some major PCBs from the blood of PCB-poisoned patients*

Peak no.	No. of Cl atoms	Substitution pattern	Elimination rate*
9-4-a†	4	2,4,5,4'(?)	+ + +
9-4-b†	4	2,5,3',4'	+ + + + +
9-4-c†	4	2,4,3',4'	+ + + +
10-4†	4	2,3,3',4'(?)	+ + + + +
11-5-a†	5	2,5,2',3',5'	+ + + +
11-5-b†	5	2,5,2',4',5'	+ + + +
11-5-c†	5	2,4,2',4',5'	+ +
12-5-a†	5	2,3,2',4',5'	+ + + + +
12-5-b†	5	2,5,2',3',4'	+ + + + +
12-5-c†	5	2,4,2',3',4'	+ + + + (?)
13-5-a†	5	2,3,6,3',4'	+ + + + +
13-5-b	5	—	+ + + + +
14-5	5	2,3,4,5,3'(?)	+ + + +
15-5-a†	5	2,4,5,3',4'	+ + +
15-5-b	5	—	+ +
16-5†	5	2,3,4,3',4'	+ + +
16-6†	6	2,4,5,2',4',5'	+
17-6	6	—	+ + + +
18-6†	6	2,3,4,2',4',5'	+
19-6†	6	2,3,4,2',3',4'	+ +
20-6†	6	2,3,4,5,3',4'	+
21-7†	7	2,3,4,5,2',4',5'	+
22-7†	7	2,3,4,5,2',3',4'	+

*The symbols + + + + +, + + + +, + + +, + + and + denote rapid, less rapid, slow, very slow and extremely slow, respectively.

†Structural assignments of these PCBs are mainly based on a comparison of published data with the GC retention indices determined for the major PCBs in toxic rice-bran oil from Taichung (see text, p. 419).

RESULTS

A gas chromatogram of PCBs in the contaminated rice-bran oil ingested by patients in Taichung is shown in Fig. 1. This chromatogram was obtained with a highly efficient glass capillary column coated with SE-30. More than fifty-five peaks can be resolved on this column. The structural assignments of most of the major peaks are shown in Table 1. These structural assignments are based on the comparison of the retention indices determined in our laboratory with those reported by Albro and his coworkers (Albro, Corbett & Schroeder, 1981; Albro, Haseman, Clemmer & Corbett, 1977), and also on the published structural identifications of PCB components in the commercial PCB preparations (Nakamura & Kashimoto, 1977; Sissons & Welti, 1971) and in the Japanese Yusho patients (Kuroki & Masuda, 1977; Masuda & Kuratsune, 1979). The identification of several of the peaks (11-5-b, 16-6 and 18-6) with the PCB components is confirmed by the coincidence of the retention times of these peaks with those of the authentic PCB reference standards. The numbers of chlorine atoms in the PCB congeners corresponding to each peak shown in Table 1 were obtained by GC/MS analysis operating on the selected ion monitoring mode.

Fig. 2 shows chromatograms of PCBs in the blood of a Taichung patient. The first blood sample (Fig. 2A) was drawn for PCB analysis about 9 months after he was poisoned, while the second (Fig. 2B) was taken about 15 months later. Comparison of Fig. 1 with Fig. 2 and also of Fig. 2A with Fig. 2B indicates that different PCB congeners were eliminated from the patient's blood at different rates. PCB components corresponding to peaks 13-5-a, 10-4, 9-4-b, 12-5-a and

Table 2. *Relative concentrations of some major PCBs in blood samples taken from patients at various intervals after PCB poisoning**

Patient no.	Blood-sampling date	Relative concentrations† of PCBs									Total PCBs in blood (ppb)
		11-5-c	15-5-a	16-5	16-6	18-6	19-6	20-6	21-7	22-7	
1	21/7/80	2·7	5·75	3·21	2·51	4·03	0·88	1·31	1·00	0·77	39
	14/4/81	2·4	3·36	1·71	2·31	3·57	0·76	1·31	1·00	0·77	29
	22/6/81	2·3	2·96	1·36	2·20	3·54	0·69	1·32	1·00	0·79	28
2	31/1/80	2·8	5·57	3·04	2·57	4·63	0·76	1·41	1·00	0·88	43
	4/6/80	2·8	4·15	1·75	2·57	4·49	0·67	1·31	1·00	0·83	39
	28/4/81	2·5	1·89	0·61	2·49	4·50	0·64	1·40	1·00	0·89	35
3	31/1/80	2·6	5·57	2·89	2·37	4·31	0·74	1·15	1·00	0·72	40
	15/7/80	2·5	4·00	1·71	2·37	4·23	0·79	1·16	1·00	0·71	35
	26/3/81	2·2	1·68	0·61	2·43	4·09	0·71	1·16	1·00	0·72	26
4	1/2/80	3·1	4·75	2·96	2·43	4·23	0·83	1·37	1·00	0·88	26
	27/5/80	3·0	3·86	1·96	2·40	4·26	0·81	1·37	1·00	0·80	22
	28/4/81	2·7	1·64	0·54	2·17	3·89	0·74	1·40	1·00	0·87	16
5	12/3/80	3·0	1·64	0·64	2·69	4·66	0·76	1·29	1·00	0·82	30
	15/7/80	2·8	0·70	—	2·60	4·44	0·62	1·24	1·00	0·76	24
	22/6/81	2·7	0·14	—	2·49	4·57	0·45	1·24	1·00	0·76	22
6	26/2/80	1·8	4·71	2·50	2·20	4·10	0·64	1·29	1·00	0·74	28
	30/9/80	2·0	3·07	1·11	2·17	4·00	0·55	1·34	1·00	0·79	24
	12/6/81	1·9	1·75	0·54	2·20	3·94	0·55	1·40	1·00	0·73	22
7	26/2/80	1·8	3·78	1·46	2·51	4·33	0·66	1·26	1·00	0·83	33
	22/6/81	1·7	0·43	0·11	2·46	3·97	0·48	1·19	1·00	0·79	22
8	14/4/80	2·6	1·11	0·39	2·40	4·03	0·45	1·29	1·00	0·77	35
	28/4/81	2·4	0·25	—	2·51	4·11	0·43	1·35	1·00	0·77	28
9	1/2/80	1·9	3·00	1·43	2·26	3·94	0·60	1·37	1·00	0·77	43
	22/6/81	1·8	0·43	0·04	2·20	4·03	0·50	1·25	1·00	0·79	31
10	4/1/80	1·6	2·57	1·46	2·17	3·94	0·64	1·34	1·00	0·77	44
	22/6/81	1·7	0·21	—	2·14	3·94	0·64	1·34	1·00	0·77	31
11	14/1/80	2·4	5·73	3·75	2·06	4·00	0·67	1·34	1·00	0·82	56
	22/6/81	2·3	2·82	1·18	2·20	4·11	0·67	1·41	1·00	0·82	44
12	1/4/80	1·9	2·21	0·93	2·09	3·09	0·57	1·22	1·00	0·73	26
	26/3/81	1·7	0·75	0·21	2·06	3·00	0·57	1·16	1·00	0·72	19
13	23/3/80	2·0	5·50	2·79	2·14	3·77	0·74	1·32	1·00	0·81	32
	5/5/81	2·0	2·50	0·93	2·11	3·74	0·69	1·32	1·00	0·76	21
14	14/1/80	2·6	6·43	3·57	2·59	4·56	0·80	1·40	1·00	0·88	95
	22/6/81	2·5	1·50	0·29	2·47	4·20	0·57	1·22	1·00	0·82	53
15	7/1/80	2·2	7·11	4·21	2·63	4·40	0·86	1·24	1·00	0·76	55
	26/5/81	2·5	4·29	1·61	2·37	4·31	0·74	1·25	1·00	0·76	42
16	22/4/80	6·7	5·21	2·35	3·60	4·60	0·50	1·43	1·00	0·80	42
	30/9/80	6·7	4·14	0·71	3·60	4·74	0·50	1·35	1·00	0·77	42
	26/5/81	7·0	2·14	0·25	3·57	4·70	0·45	1·34	1·00	0·76	33
17	1/2/80	6·8	8·54	3·61	4·09	5·66	0·69	1·54	1·00	0·86	110
	14/4/81	6·7	3·18	0·64	4·31	5·43	0·52	1·53	1·00	0·82	87

*The outbreak of poisoning occurred in March–April 1979.
†Relative to peak 21-7 (= 1·00).

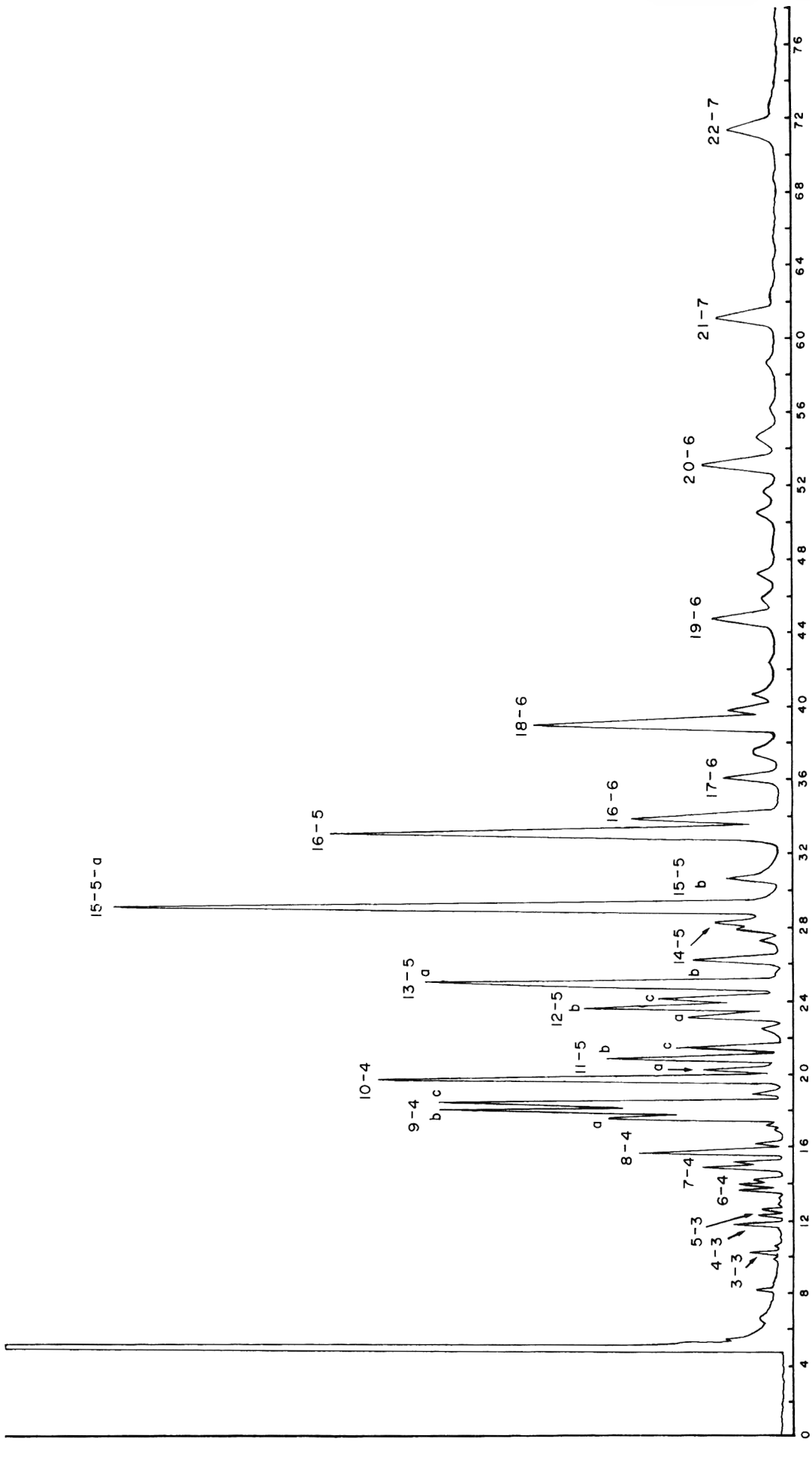

Fig. 1. Separation of PCBs in the toxic rice-bran oil obtained from Taichung, using a 30 m × 0·25 mm ID glass capillary column coated with SE-30 and operated at 200°C.

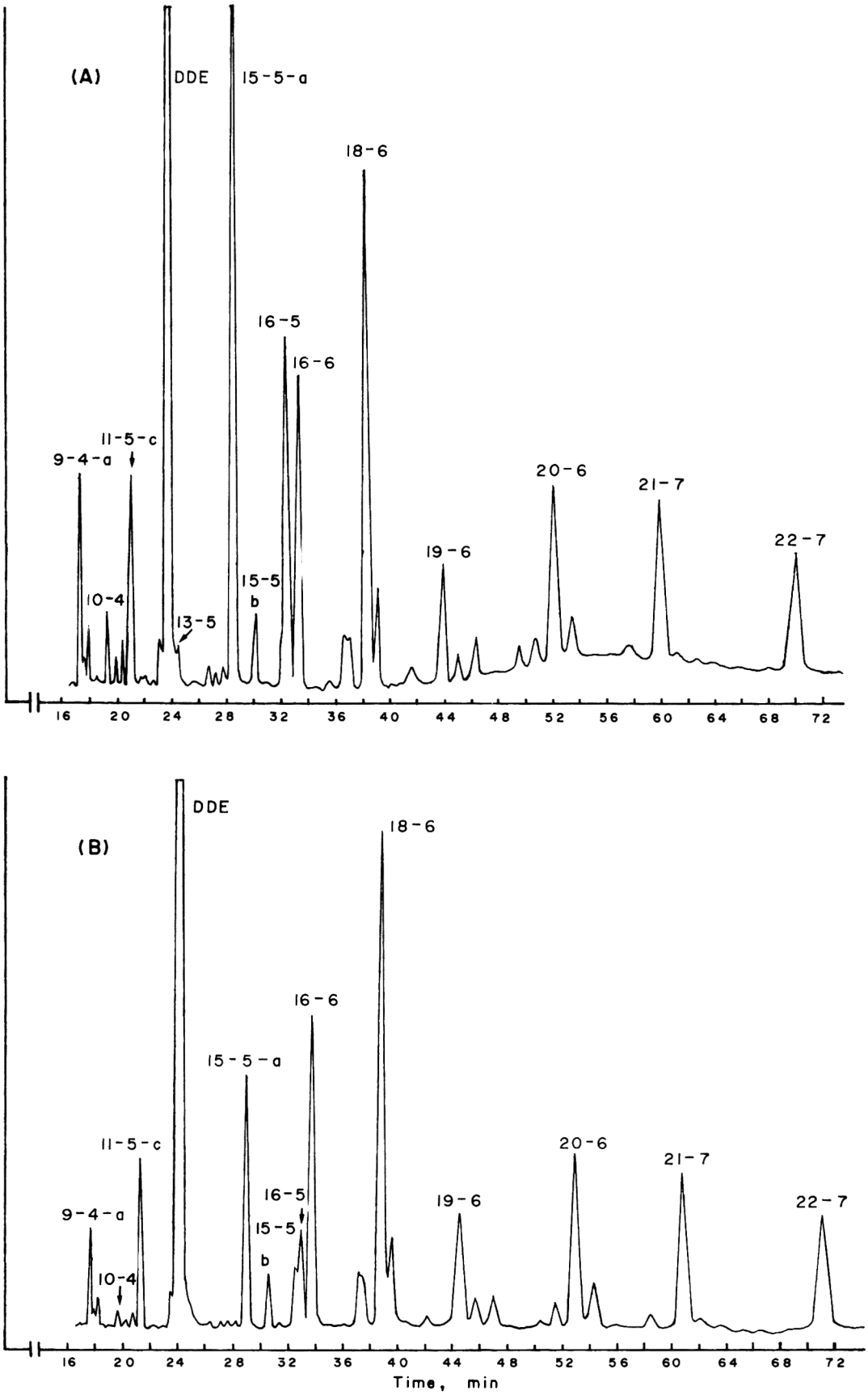

Fig. 2. Gas chromatograms of PCBs in blood samples taken from a Taichung patient (A) on 1 February 1980 and (B) on 28 April 1981. The column and operating temperature are as described for Fig. 1.

-b and 13-5-b were rapidly removed from the blood, while those corresponding to peaks 9-4-c, 11-5-a and -b, 14-5, 17-6 and possibly 12-5-c (covered by DDE) were less rapidly eliminated from the blood. Peaks 9-4-a, 15-5-a and 16-5 are PCBs that were removed from the blood at slower rates, while PCBs corresponding to peaks 11-5-c, 15-5-b, 16-6, 18-6, 19-6, 20-6, 21-7 and 22-7 were removed only very slowly or scarcely at all and would be expected to remain in the patient's body for a long time. These observations on the comparative rates of elimination of individual PCBs from the patient's blood are also applicable to other patients and are summarized in Table 1.

The data on the relative concentrations of some major PCBs in blood taken from 17 patients at two or three intervals after poisoning are tabulated in Table 2. The levels of total PCBs in the blood samples from these patients are also included in the table, for reference. The relative concentrations of PCBs for peaks 11-5-c, 15-5-a, 16-5, 16-6, 18-6, 19-6, 20-6, 21-7 and 22-7 were calculated by dividing the area of each peak (standardized to peak 21-7) by a factor of 0·09, 0·28, 0·28, 0·35, 0·35, 0·42, 0·68, 1·00 and 0·95, respectively. These division factors (ECD response factors) are the relative area responses of the ECD for these PCB components calculated in our laboratory. They may have some degree of inaccuracy because only a few of the authentic PCB reference standards for these peaks were available for accurate calculation. In spite of this, the estimation of comparative ease of elimination (Table 1) and the calculations of the half-lives of two PCB isomers in the blood (Table 3) from the data in Table 2 should not be subject to the same kind of inaccuracy. This is because the same proportion of change in the relative concentration of an individual PCB at two different time points will be obtained even when different division factors are used for the calculation of the relative concentration of that PCB component; therefore, the estimation of elimination rates should not be affected.

Table 3. *Terminal half-lives of 2,4,5,3',4'-pentachlorobiphenyl (15-5-a) and 2,3,4,3',4'-pentachlorobiphenyl (16-5) in the blood of PCB-poisoned patients*

Patient no.	Half-life (months)	
	15-5-a	16-5
1	11·3	9·0
2	9·6	6·4
3	8·1	6·2
4	9·6	6·1
5	4·1	—
6	10·8	6·8
7	5·2	4·3
8	5·9	—
9	5·8	3·3
10	4·8	—
11	17·8	9·8
12	7·6	5·6
13	12·8	9·1
14	8·2	4·6
15	24·1	12·4
16	10·2	4·3
17	9·9	5·6
Mean ± 1 SD	9·8 ± 5·0	6·7 ± 2·5

*The patient numbering corresponds to that used in Table 2.

In Table 2, the component 2,3,4,5,2',4',5'-heptachlorobiphenyl (peak 21-7) was chosen as the basis for the standardization of each individual PCB. This peak was chosen because it is a highly chlorinated PCB containing no adjacent unsubstituted carbon atoms and therefore it should not be metabolized to any appreciable extent (Matthews & Tuey, 1980). On the assumption that peak 21-7 is a non-metabolized PCB and is very resistant to excretion from the body, one can use the data in Table 2 to calculate the comparative rates of elimination of PCBs from the patients' blood. The terminal half-lives of 2,4,5,3',4'-pentachlorobiphenyl (peak 15-5-a) and 2,3,4,3',4'-pentachlorobiphenyl (peak 16-5) in the blood were calculated from the data in Table 2, and are listed in Table 3. The half-lives of other PCBs in the blood were not calculated because they were either too long or too short to calculate or because the concentrations of these PCB components in the blood were too small to measure accurately.

Table 3 shows that, for every patient, 2,4,5,3',4'-pentachlorobiphenyl was more slowly eliminated than 2,3,4,3',4'-pentachlorobiphenyl, but the variation among the patients was quite large. While the half-lives for the elimination of 2,4,5,3',4'-pentachlorobiphenyl from the blood varied from 4·1 to 24·1 months with a mean value of 9·8 ± 5·0 months, they varied from 3·3 to 12·4 months with a mean value of 6·7 ± 2·5 months for 2,3,4,3',4'-pentachlorobiphenyl.

With the exception of patients 16 and 17, who were from Changhwa, all the patients listed in Tables 2 and 3 were from Taichung. Chromatograms of PCBs in the blood of a Changhwa patient are shown in Fig. 3 for comparison with those from the Taichung patient (Fig. 2). These indicate that the Changhwa patient had higher intensities of 11-5-c and 9-4-a than did the Taichung patient. These differences were also observed for other patients from Taichung and Changhwa and probably reflected the presence of higher percentages of these two components in the toxic oil ingested by the patients in Changhwa than in the oil ingested in Taichung, although no sample of the toxic oil that caused PCB poisoning in Changhwa has ever been available for analysis (Chen *et al.* 1981) to confirm this supposition. The relative concentrations of 11-5-c for both Taichung and Changhwa patients are shown in Table 2, but the concentrations of 9-4-a for these patients are not shown because with this PCB the ECD response factor is small (about 0·06 compared to 1 for 21-7) and consequently the area for the Taichung patients is generally too small to permit an accurate calculation of the relative concentration.

It should be pointed out that peak 16-5 (a pentachlorobiphenyl) in both Fig. 2A and Fig. 3A contains a minor component of hexachlorobiphenyl on its left shoulder. This is more clearly evident in Figs 2B and 3B, the non-metabolized hexachlorobiphenyl being more prominent when most of the pentachlorobiphenyl has been metabolized and eliminated from the blood. As shown in Fig. 3B, this hexachlorobiphenyl (the left one of the doublet) is of the same intensity as the pentachlorobiphenyl (16-5).

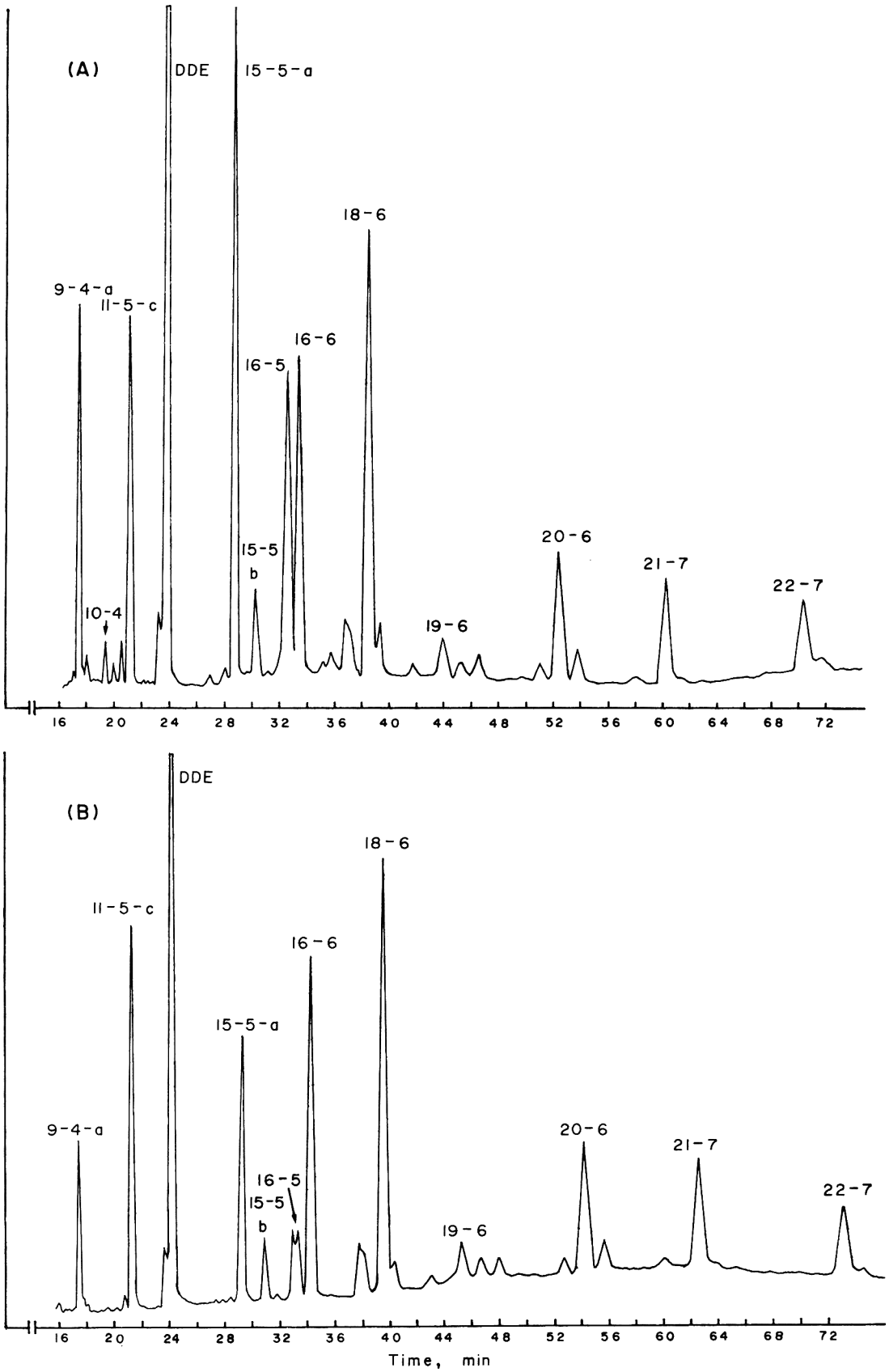

Fig. 3. Gas chromatograms of PCBs in blood samples taken from a Changhwa patient (A) on 1 February 1980 and (B) on 14 April 1981. The column and operating temperature are as described for Fig. 1.

DISCUSSION

Instead of absolute concentrations, relative concentrations of PCBs, expressed in relation to 2,3,4,5,2',4',5'-heptachlorobiphenyl (21-7), were used in the estimation of comparative rates of elimination of individual PCBs from the blood. This was because no internal standard was used in our quantification of PCBs and therefore a significant variation in PCB levels between repeated analysis was to be expected. The difficulty of accurate quantification of individual PCBs was resolved by relating PCB concentrations to a non-metabolized PCB, so that a decrease in the relative concentration of a given PCB after a certain time would reflect the actual fall in its concentration. The assumption that the heptachlorobiphenyl (21-7) is not metabolized to any appreciable extent is not only predictable from its structure (Matthews & Tuey, 1980) but is also substantiated by the absolute quantification of this PCB in many patients, indicating that the concentration of this heptachlorobiphenyl did not change significantly with time after poisoning.

Table 1 shows that, with the exception of 17-6, all the PCBs with six or more chlorine atoms were very slowly or extremely slowly eliminated. It has been well established that excretion of PCBs depends primarily on their rates of metabolism (Matthews & Anderson, 1975) and therefore the slowly eliminated PCBs are likely also to be those that are slowly metabolized. The slowly eliminated hexa- and heptachlorobiphenyls in Table 1 either do not have adjacent unsubstituted carbon atoms (16-6 and 21-7) or have one pair (18-6, 20-6, 22-7) or two pairs (19-6) of adjacent unsubstituted carbon atoms at *ortho–meta* positions. This suggests that when PCBs have six or more chlorine atoms in the biphenyl ring, the presence of two adjacent unsubstituted carbon atoms at the *ortho–meta* positions does not markedly facilitate metabolism. The presence of two pairs of unsubstituted carbon atoms at the *ortho–meta* positions, as in the case of 19-6, seems to facilitate elimination to some extent. With the exception of 19-6, the non-metabolized PCBs mentioned above were also reported by Japanese scientists to be retained in patients with Yusho (Kuroki & Masuda, 1977). It should be noted that these slowly metabolized PCBs all have only one or two chlorine atoms in the *ortho* position. PCBs with three or four *ortho* chlorines may be retained in the body for shorter periods; Gage & Holm (1976), for example, reported that 2,3,4,2',4',6'-hexachlorobiphenyl was lost rapidly from fat in an animal study.

Among the twelve pentachlorobiphenyl isomers listed in Table 1, the compounds with one pair of unsubstituted carbon atoms at *ortho–meta* positions (i.e. 11-5-c and 15-5-a) are eliminated more slowly than those with unsubstituted carbon atoms at *meta–para* positions (i.e. 11-5-a and -b, 12-5-a and -b, 13-5-a and possibly 14-5). The former are also more slowly eliminated than those with two pairs of unsubstituted carbon atoms at the *ortho–meta* positions (i.e. 12-5-c and 16-5). A comparison of the data on the elimination rates and chlorine substitution positions for the tetrachlorobiphenyls in Table 1 also indicates that PCBs with two adjacent unsubstituted carbon atoms at the *meta–para* positions (i.e. 9-4-b and 10-4) are more rapidly eliminated than those with adjacent unsubstituted carbon atoms only at the *ortho–meta* positions (i.e. 9-4-c and possibly 9-4-a). These observations that two adjacent unsubstituted carbon atoms at the *meta–para* positions facilitate the metabolism of PCBs are consistent with the findings in experimental animal studies reported by other workers (Billings & McMahon, 1978; Matthews & Tuey, 1980).

In summary, this work on the elimination of some major PCBs from the blood of PCB-poisoned patients indicates that those with adjacent unsubstituted carbon atoms at *meta–para* positions will be rapidly eliminated from the blood, while PCBs with adjacent unsubstituted carbon atoms at *ortho–meta* positions will be less rapidly or only slowly eliminated. The data presented here also indicate that hexa- and heptachlorobiphenyls with adjacent unsubstituted carbon atoms at the *ortho–meta* positions are eliminated very slowly. Since it has been well established that excretion of PCBs depends primarily on their rates of metabolism (Matthews & Anderson, 1975; Matthews & Tuey, 1980), these differences in rates of elimination of PCBs should be a reflection of the differences in their rates of metabolism in man.

Acknowledgements—The authors wish to thank the National Science Council of the Republic of China for financial support for this study. One of us (PHC) is also grateful to Tjing-Ling Medical Foundation for a Research Chair Award during the time this work was conducted.

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Fd Chem. Toxic. Vol. 20, pp. 427 to 432, 1982
Printed in Great Britain

0278-6915/82/040427-06$03.00/0
Pergamon Press Ltd

EVALUATION, USING *SALMONELLA TYPHIMURIUM*, OF THE MUTAGENICITY OF SEVEN CHEMICALS FOUND IN COSMETICS

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(*Received 5 January 1982*)

Abstract—Six chemicals used as ingredients in cosmetics were evaluated for mutagenic activity in *Salmonella typhimurium*. Two of these ingredients, *trans*-4-phenyl-3-butene-2-one and 2,2',4,4'-tetrahydroxybenzophenone, were mutagenic in the presence of rat liver S-9 towards strains TA100 and TA1537 respectively. An impurity found in some cosmetic products, *N*-nitrosodiethanolamine, was mutagenic to *S. typhimurium* strains TA1535 and TA100 in the presence of hamster-liver S-9 but not rat-liver S-9.

INTRODUCTION

Current federal laws in the United States give various regulatory agencies the authority to require chemical manufacturers to submit to the government toxicological data necessary to evaluate the safety of almost all types of commercial chemicals. Chemicals used exclusively as cosmetics ingredients, however, are not subject to any such requirements. We therefore initiated a pilot project to determine whether or not chemicals used in cosmetics may have mutagenic activity. In addition, *N*-nitrosodiethanolamine (NDELA), which is a carcinogen that has been found in several cosmetic products as an impurity, was evaluated because there are conflicting reports in the literature concerning the mutagenicity of this chemical.

EXPERIMENTAL

Test chemicals. *Trans*-cinnamaldehyde (CAS No. 104-55-2), 3,4-dihydrocoumarin (hydrocoumarin, CAS No. 119-84-6) and *trans*-4-phenyl-3-butene-2-one (TPBO, CAS No. 1896-62-4) are fragrance ingredients. They were selected for testing because they have been reported to be strong sensitizers (Opdyke, 1973, 1974 & 1979). Methyl-*p*-hydroxybenzoate (methylparaben, CAS No. 99-76-3) is used in cosmetics as a preservative. 2,2',4,4'-Tetrahydroxybenzophenone (THBP, CAS No. 131-55-5) is a UV-light absorber; it is used as a sunscreen for humans and to prevent the decomposition of cosmetic formulations when exposed to sunlight. All of these chemicals, labelled as 99% pure, were purchased from the Aldrich Chemical Co., Milwaukee, WI. Additional samples of THBP, under the trade name "Uvinul D-50", were obtained as gifts from the GAF Corp., New York, NY, and an additional sample of TPBO, under the commercial name "benzylidene acetone", was obtained from Fritzsche, Dodge, & Olcott, Inc., New York, NY. Isoamyl-*p*-(dimethylamino)benzoate (padimate A, CAS No. 21245-01-2), which has been used as a sunscreen, was obtained as a gift from Van Dyk & Co., Belleville, NJ, under the trade name "Escalol 506". NDELA (CAS No. 1116-54-7), which is found as a contaminant in a variety of cosmetic products (Fan, Goff, Song *et al.* 1977; Klein, Girard, de Smedt *et al.* 1981), was a generous gift from Dr. L. Wallcave of the Eppley Institute for Research in Cancer, Omaha, NE.

Plate incorporation assays. The bacteria used in this work were histidine-requiring strains of *S. typhimurium* described by Ames, McCann & Yamasaki (1975b), and were obtained from Professor B. N. Ames (Berkeley, CA). Plate incorporation assays were performed as described by Ames *et al.* (1975b) except that the base agar contained only 0·5% glucose. Unless otherwise stated, 100 μl Aroclor 1254-induced rat liver S-9 was used in each assay.

With the exception of NDELA, the chemicals were tested in the plate incorporation assay as follows. A mutagenesis test was performed using four to six doses separated by factors of ten, the highest dose being 10 mg/plate. Such a test was performed with four *S. typhimurium* strains (TA1535, TA1537, TA98 and TA100) with and without metabolic activation. The test was then repeated with four to six doses separated by approximately half-log intervals (e.g., 3, 1, 0·3 and 0·1 mg), the highest dose being 3 mg or a minimal toxic dose determined in the first experiment. If the first two experiments appeared to give positive results, additional experiments were performed to confirm the positive response and obtain dose-response curves.

Positive control compounds used in screening tests without S-9 mix were ethylmethane sulphonate (TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98), nitrofurantoin (TA100) and sodium azide (TA1535 and TA100). In the presence of S-9 mix, 2-fluorenylacetamide was used as the positive control with TA98 and 2-anthramine was used with the other three strains of *S. typhimurium*. All platings were performed at least in duplicate at all doses in each ex-

Abbreviations: NDELA = *N*-Nitrosodiethanolamine; PBS = Phosphate-buffered saline; SGM = Suspension growth medium; TPBO = *Trans*-4-phenyl-3-butene-2-one; THBP = 2,2',4,4'-Tetrahydroxybenzophenone.

periment. Test and positive control chemicals were dissolved in dimethylsulphoxide (DMSO). Each culture was checked for sensitivity to crystal violet (Ames *et al.* 1975b) and, where appropriate, resistance to 10 μg ampicillin on discs (BBL Microbiology Systems, Cockeysville, MD).

In testing NDELA, we used only *S. typhimurium* strains carrying the base-pair substitution mutation *hisG46*, because we did not expect this chemical to induce frameshift mutations. Since preliminary testing indicated that NDELA is not mutagenic when rat liver S-9 is used, hamster liver S-9 was also used in our experiments with this chemical. The solvent for NDELA was sterile distilled water.

The procedures used for preparation of S-9 fractions from Aroclor 1254-induced male Sprague–Dawley rats and male Syrian golden hamsters have been previously described (Prival, King & Sheldon, 1979).

Liquid suspension assay. THBP was also tested in a quantitative liquid suspension assay. The following media were used for this assay: suspension growth medium (SGM) which is Vogel-Bonner medium E (Vogel & Bonner, 1956) supplemented with 0·5% glucose, 2·5 μM biotin, 0·5 mM-L-histidine, and 1 g histidine assay medium (Difco Laboratories, Detroit, MI) per litre. (Histidine assay medium is a complex mixture of nutrients, including amino acids but lacking histidine.) SGM-H is SGM without histidine (SGM-H); SGM concentrated three-fold (3 × SGM); SGM agar and SGM-H agar which both contain 1·5% agar; phosphate-buffered saline (PBS) which is 0·85% NaCl containing 0·01 M-potassium phosphate buffer, pH 7·0.

The assay was performed by mixing 2 ml 3 × SGM, approximately 2×10^9 log-phase cells grown in SGM and resuspended in PBS, 1·5 ml S-9 mix (containing 0·3 ml S-9/ml of S-9 mix) and 0·15 ml THBP dissolved in DMSO, in a total volume of 6 ml. This mixture was shaken at 37°C in a 125-ml Erlenmeyer flask for 2 hr. The cells were then washed and resuspended in 0·7 ml PBS, and 0·2 ml of the cell suspension was plated in duplicate on SGM-H agar plates to determine the number of revertants. An aliquot was also diluted by 10^6 in PBS and 0·1 ml was plated in duplicate on SGM agar plates to determine the number of viable cells. The plates were then incubated at 37°C and the colonies were counted by hand.

RESULTS

Four of the cosmetics ingredients tested, 3,4-dihydrocoumarin, *trans*-cinnamaldehyde, methyl-*p*-hydroxybenzoate and isoamyl-*p*-(dimethylamino)benzoate, gave no indication of mutagenic activity in our assays.

The sample of TPBO obtained from Aldrich, which was the sample used for the initial screening, was mutagenic in strain TA100 in the presence of S-9, as shown in Table 1. To determine whether or not this activity could have been due to the presence of an impurity that might not be present in samples of this chemical used in cosmetics, we also tested a sample obtained from Fritzsche, Dodge, & Olcott, Inc., which is a supplier to cosmetics companies. The results obtained with this sample were similar to those obtained with the Aldrich chemical (Fig. 1). In addition, the Aldrich sample of TPBO was recrystallized by H.M. Davis of the Division of Cosmetics Technology, Food and Drug Administration, and then retested for mutagenic activity. As shown in Fig. 1, the mutagenic activity of this purified sample was similar to that of the original. Since we suspected that TPBO might be sensitive to UV light, we tested it in a room using only red light for illumination and then incubated the plates in the dark. Under these conditions, no difference in mutagenic activity was observed compared to that obtained with the usual fluorescent lighting in our laboratory. Therefore, the mutagenic activity of this compound is not a result of a chemical alteration induced by UV light.

THBP did not have significant mutagenic activity when tested with strains TA1535 or TA98 (Table 1). With TA100, a marginal response of approximately a doubling was obtained at 300 μg/plate in the presence of S-9. When strain TA1537 was used, THBP

Table 1. *Mutagenicity of TPBO and THBP in* S. typhimurium

Chemical	Concn (μg/plate)	No. of revertants/plate							
		TA1535		TA1537		TA98		TA100	
		−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9
TPBO (Aldrich)	0	17	13	5	8	24	47	128	126
	10	10	16	9	8	34	47	145	152
	30	16	16	7	11	26	56	117	176
	100	12	12	6	12	29	54	140	229
	300	14	19	7	15	33	45	140	541
	1000	11	13	10	11	13	46	122	571
	3000	0	2	0	3	0	11	0	179
THBP (Aldrich)	0	17	13	5	8	24	47	128	126
	3	20	10	—	—	22	46	—	—
	10	—	11	—	—	32	53	—	—
	30	8	22	8	15	24	60	150	180
	100	14	21	7	21	23	66	157	199
	300	8	20	1	41	6	62	118	236
	1000	0	0	0	0	0	17	6	190

TBPO = *Trans*-4-phenyl-3-butene-2-one THBP = 2,2',4,4'-Tetrahydroxybenzophenone

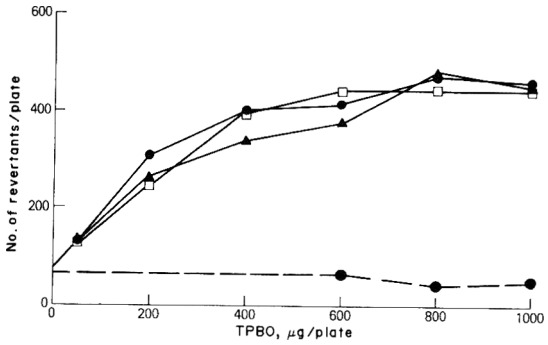

Fig. 1. Mutagenic activity of *trans*-4-phenyl-3-butene-2-one (TPBO) in *S. typhimurium* strain TA100. Data are from plate incorporation assays using 50 μl Aroclor 1254-induced rat liver S-9 per plate. TPBO from Aldrich Chemical Co. (●); recrystallized TPBO from Aldrich Chemical Co. (▲); TPBO from Fritzsche, Dodge, & Olcott, Inc. (□); solid lines, with S-9 mix; broken line, without S-9 mix.

appeared to be mutagenic in the presence of rat liver S-9 mix (Table 1). Aroclor 1254-induced hamster liver S-9 was also capable of activating THBP to a mutagen on strain TA1537 (data not shown).

To confirm that the colonies observed in the presence of THBP were *his*$^+$ revertants, we picked 28 colonies from a plate treated with 500 μg THBP in the presence of 150 μl rat liver S-9. Cells from these colonies were streaked onto minimal agar plates supplemented with biotin, but not containing histidine. The fact that cells from all 28 of the colonies grew well after incubation at 37°C demonstrates that they were all the result of mutation to histidine independence.

Two samples of THBP obtained from GAF Corp., a supplier to the cosmetics industry, were similar in their mutagenic activity to the Aldrich sample we originally tested (Fig. 2). In addition, when the Aldrich sample of THBP was recrystallized by H. M. Davis, we found that the mutagenic activity was not significantly changed (Fig. 2). Further confirmation of mutagenicity was obtained by using a quantitative liquid suspension assay with the Aldrich sample, the recrystallized Aldrich sample and one of the GAF samples of THBP (Table 2).

NDELA was not mutagenic under our usual test conditions with strain TA1535 or TA100 with or without rat liver S-9 (data not shown). However, when hamster liver S-9 was used instead of rat liver S-9, mutagenic activity was observed (Table 3).

We also found that NDELA is mutagenic to the other commonly used base-pair substitution *S. typhimurium* strains G46 and TA1530 in the presence of hamster liver S-9 (data not shown). Hesbert, Lemonnier & Cavelier (1979) reported that NDELA is mutagenic to strains TA1535 and TA100 even when no S-9 was used. We found no mutagenic activity when up to 15 mg NDELA was tested in the absence of S-9 on strains TA1535, TA100 (Table 3), TA1530 or G46 (data not shown).

DISCUSSION

Methyl-*p*-hydroxybenzoate was previously reported to be negative in a rat bone-marrow cytogenetic

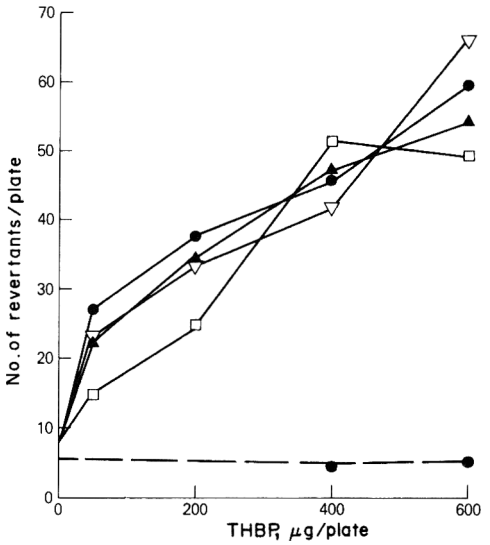

Fig. 2. Mutagenic activity of 2,2',4,4'-tetrahydroxybenzophenone (THBP) in *S. typhimurium* strain TA1537. Data are from plate incorporation assays using 150 μl Aroclor 1254-induced rat liver S-9 per plate. THBP from Aldrich Chemical Co. (●); recrystallized THBP from Aldrich Chemical Co. (▲); THBP samples from GAF (□) and (▽); solid lines, with S-9 mix; broken line, without S-9 mix.

Table 2. *Suspension assay of THBP with* S. typhimurium *strain TA1537, using Aroclor 1254-induced rat liver S-9*

THBP sample	THBP (mg/ml)	No. of revertants/ml*	cfu/ml × 10^{-8}*	No. of revertants/10^8 cfu
	0·0	40	36	1·1
Aldrich	0·025	10	16	0·6
	0·1	20	6·8	2·9
	0·2	108	5·8	19
	0·3	348	7·0	50
Aldrich (recrystallized)	0·3	415	6·6	63
GAF	0·3	368	12	31

THBP = 2,2',4,4'-Tetrahydroxybenzophenone cfu = Colony-forming units

*As determined in 0·7 ml resuspended culture volume.

Table 3. *Mutagenicity of NDELA in* S. typhimurium *in the presence and absence of hamster liver S-9*

Chemical	Concn (mg/plate)	No. of revertants/plate			
		TA1535		TA100	
		−S-9	+S-9	−S-9	+S-9
NDELA	0	15	9	190	227
	5	11	293	207	665
	10	12	635	188	1019
	15	16	644	171	1334
Na azide	0·0013	207	—	614	—
Nitrofurantoin	0·0005	—	—	1885	—

*+S-9 indicates the presence of S-9 mix containing 150 μl Aroclor 1254-induced hamster liver S-9/plate.

assay, a dominant lethal test in rats and a host-mediated assay in mice using *S. typhimurium* strains G46 and TA1530 as well as *Saccharomyces cerevisiae* strain D3 (Litton Bionetics, Inc., 1974). In a more recent study, this compound has been reported to induce chromosomal aberrations, including exchanges and rings, in Chinese hamster lung cells when tested *in vitro* in the presence of rat liver S-9 (Matsuoka, Hayashi & Ishidate, 1979). We have found no mutagenic activity in our assays using *S. typhimurium*.

Cinnamaldehyde was previously reported to be non-mutagenic when tested in *S. typhimurium* strains TA98 and TA100 in a "preincubation" assay using rat liver S-9 (Sasaki & Endo, 1978). It was however reported to be a weak mutagen in *Drosophila melanogaster* (Venkatasetty, 1972). We found no indication of mutagenic activity in four *S. typhimurium* strains under our test conditions.

Two of the six cosmetic ingredients tested were mutagenic to *S. typhimurium*. TPBO was mutagenic only to strain TA100 and only in the presence of a mammalian metabolic activation system.

THBP was clearly mutagenic only in strain TA1537. Other chemicals, including certain acridines (Brown, Firth & Yielding, 1980; Kalinowska & Chorazy, 1980; McCann, Choi, Yamasaki & Ames, 1975) anthraquinones and benzanthrones (Brown & Brown, 1976; Brown & Dietrich, 1979), and quinacrine (McCoy, Rosenkranz, Petrullo *et al.* 1981), have also been reported to be specific for strain TA1537. The specificity of THBP for strain TA1537 may indicate that this chemical causes frameshift mutations by intercalating between DNA bases without covalent binding. We believe that particular caution should be taken in extrapolating from mutagenicity to potential carcinogenicity for this compound. Clayson (1980) has discussed the possibility that mutagenicity may not correlate well with carcinogenicity for intercalating agents that do not bind covalently to DNA.

NDELA, a contaminant in some cosmetic products, was mutagenic at high doses in the presence of hamster liver S-9, but not rat liver S-9. This same species specificity has previously been reported for the activation of other aliphatic and hydroxylated aliphatic nitrosamines, including *N*-nitrosodimethylamine (Prival & Mitchell, 1981), *N*-nitrosodiethylamine (Prival *et al.* 1979), *N*-nitrosomethyl-*n*-propylamine and *N*-nitrosodi-*n*-propan-2-olamine (Bartsch, Malaveille & Montesano, 1976). Rao, Young, Lijinsky & Epler (1979) found NDELA to be non-mutagenic in the *Salmonella* assay in the presence of rat liver S-9 at doses up to 2 mg/plate. Gilbert, Fabry, Rollmann *et al.* (1981) also reported negative results with up to 10 mg NDELA/plate, with or without rat-liver S-9. However, Hesbert *et al.* (1979) reported that this compound gives a linear dose-response in strains TA100 and TA1535 in the presence or absence of mouse liver S-9. Our findings are consistent with those of Rao *et al.* (1979) and Gilbert *et al.* (1981) in that we also found that NDELA is not mutagenic in the presence of rat liver S-9. However, our results contradict those of Hesbert *et al.* (1979) because we observed no mutagenic activity in the absence of S-9. Since our data and those of Rao *et al.* (1979) and Gilbert *et al.* (1981) indicate the lack of mutagenic activity in the absence of S-9, and since the mutagenicity of other similar aliphatic or hydroxylated aliphatic nitrosamines requires mammalian metabolic activation, we conclude that such activation is required for the mutagenicity of NDELA.

The requirement of NDELA for a specific type of mammalian liver S-9 is not considered to be related to any species specificity for toxicological effects *in vivo*. NDELA is carcinogenic to both rats (Druckrey, Preussmann, Ivankovic & Schmahl, 1967; Lijinsky, Reuber & Manning, 1980) and hamsters (Hilfrich, Schmeltz & Hoffmann, 1978; Pour & Wallcave, 1981). This is similar to the case of *N*-nitrosodimethylamine which is mutagenic in the *S. typhimurium* plate assay in the presence of hamster liver S-9 but not rat liver S-9 (Prival & Mitchell, 1981) although it is carcinogenic in at least 12 animal species, including the rat (IARC, 1978).

Two of the six cosmetic ingredients we tested were mutagenic to *S. typhimurium*. Other cosmetic ingredients, particularly hair dye chemicals, have previously been reported to be mutagens (Ames, Kammen & Yamasaki, 1975a; Blijleven, 1977; Gocke, King, Eckhardt & Wild, 1981; Green & Pastewka, 1980; Muzzall & Cook, 1979; Palmer, Denunzio & Green, 1977; Prival, Mitchell & Gomez, 1980; Searle, Hamden, Venitt & Gyde, 1978) and carcinogens (Ito, Hiasa, Koniski & Marugami, 1969; Ward, Stinson, Hardisty *et al.* 1979). In view of the widespread and high-level exposure of the population to cosmetic chemicals and the ability of many of these chemicals

to penetrate the skin, it would seem that more thorough toxicological evaluation of both new and existing cosmetic ingredients is warranted.

Acknowledgements—We thank H. M. Davis for recrystallizing the samples of THBP and TPBO and H. J. Eiermann for useful discussions about the chemicals investigated in these experiments.

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Fd Chem. Toxic. Vol. 20, pp. 433 to 440, 1982
Printed in Great Britain.

0278-6915/82/040433-08$03.00/0

ALTERATIONS OF RENAL TISSUE STRUCTURE DURING A 30-DAY GAVAGE STUDY WITH NITRILOTRIACETATE

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(*Received 29 December 1981*)

Abstract Nitrilotriacetate (NTA), as the monohydrated trisodium salt, was administered by gavage to male Sprague–Dawley rats at levels of 0, 0·73 or 7·3 mmol/kg body weight/day for periods of up to 30 days. Two animals from each of the groups were killed 24 hr after dosing on day 9, 13, 16, 20, 23, 27 or 30. Cytoplasmic vacuolation and hyperplasia of the proximal convoluted tubules were the most prominent alterations observed by light microscopic examination of kidney tissue from both groups of NTA-treated rats. The number and severity of the lesions was greater in the high-dose group and in this group, erosion and hyperplasia of the pelvic transitional epithelium were also noted. The results of this study suggest that NTA-associated urinary tract lesions develop in a sequential pattern and that the rate and extent of these lesions is dose dependent.

INTRODUCTION

Nitrilotriacetate (NTA) is a highly effective chelating agent that has been found to induce urinary tract toxicity and tumour formation in rats and mice only when administered at relatively high dose levels (Lijinsky, Greenblatt & Kommineni, 1973; National Cancer Institute, 1977). In recent years the mechanism underlying the tumorigenic action of NTA has been a focus of study in our laboratory. The decision to pursue this research was based on several characteristics of NTA that distinguish it from most other carcinogens: NTA has been found to be non-mutagenic (Kramers, 1976; Stine & Hardigree, 1971) and, following absorption, it does not undergo any detectable biotransformation, except for cation exchange (Budny, 1972; Budny & Arnold, 1973; Michael & Wakim 1971); The development of tumours in rats and mice dosed with NTA has been restricted to the urinary tract; finally, manifestations of NTA toxicity were observed in urinary tract tissue only when NTA was ingested at levels equivalent to or greater than 0·15% of the diet (*c.* 0·55 mmol/kg body weight/day; Anderson, Alden & Merski, 1982; Nixon, 1971; Nixon, Buehler & Niewenhuis, 1972). On the basis of this and other information it has been suggested that NTA-associated urinary tract tumour formation is an epigenetic phenomenon which occurs as a final step in a series of pathological changes that take place only if NTA is ingested for extended periods (*c.* ⩾ 18months) at levels that are toxic to the urinary tract tissues (Anderson *et al.* 1982).

Previous work (Merski, 1981) has shown that cytoplasmic vacuolation of the cells of the proximal convoluted tubules (PCTs) was the only lesion observed in the renal tissue of rats that were given by gavage a single dose of 7·3 mmol NTA/kg body weight. The vacuolation develops within 6 hr after treatment and may last for up to 72 hr. If NTA dosing is continued, this type of lesion has been found to persist and to be followed by the development of a variety of other degenerative and proliferative lesions of the urinary tract (Alden, Kanerva, Anderson & Adkins, 1981). The present study was carried out to provide information on possible relationships between PCT cell vacuolation and subsequent changes in renal structure, as well as on the frequency and types of lesions observed at the dose levels of NTA used.

Abbreviations: NTA = Trisodium nitrilotriacetate monohydrate; PCT = proximal convoluted tubule.

EXPERIMENTAL

Animals. Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were used in this study. Following receipt the animals were housed individually in stainless-steel wire-bottomed cages and held in quarantine for 3 days. At the end of this period the rats were weighed and randomly assigned to one of three treatment groups. The weights of the rats at the beginning of the study ranged from 100 to 119 g. All of the rats were weighed twice during each week of the study. Free access to ground Purina rat chow (Ralston Purina Co. Inc., St Louis, MO) and distilled water was permitted both prior to and during the experimental period.

Dosing. All NTA solutions were prepared by dissolving commercial grade NTA, as the monohydrated trisodium salt ($Na_3NTA.H_2O$; Monsanto Chemical Co., St. Louis, MO), in deionized distilled water. The pH of these solutions was adjusted to 8·5 by the addition of concentrated HCl. Depending on the dose to be administered, the final concentration of the NTA solutions was either 0·9 or 0·09 mmol/ml. NTA was administered by gavage at levels of either 7·3 or 0·73 mmol/kg body weight. These doses are roughly equivalent to those used in previous subchronic NTA feeding studies that have been shown to induce cytoplasmic vacuolation in PCT cells (Nixon, 1971; Nixon *et al.* 1972). Control animals were given equivalent volumes of deionized distilled water. All of the sol-

utions were administered daily by gavage for up to 30 days. Adjustments in the amount of NTA administered were made twice a week to take account of changes in the animals' weight.

Sample preparation. Two rats from each treatment group and the control group were killed approximately 24 hr after dosing on day 9, 13, 16, 20, 23, 27 or 30 of treatment. At each of these time points the rats were anaesthetized with pentobarbital (50 mg/kg body weight) or ether and the kidneys were quickly excised. The kidneys were sectioned longitudinally into approximately equal ventral and dorsal halves. These pieces were placed in a fixative solution consisting of 4% formaldehyde and 2% glutaraldehyde in 0·1 M-phosphate buffer (pH 7·3) for at least 24 hr before being dehydrated and embedded by standard techniques. Sections for light microscopy were prepared from the cut face of each kidney half and stained with haematoxylin and eosin. One such section from each kidney half was examined at a magnification of × 125.

Classification of lesions. Grading of the cytoplasmic vacuolation of the PCT cells was based on three categories of this alteration previously described (Merski, 1981); a brief description of these changes is presented in the Results. The categories of hyperplastic lesions were based on those previously reported by Alden *et al.* (1981). The values reported in Table 1 for the vacuolar and hyperplastic lesions refer to the number of tubule cross-sections observed with that particular alteration.

RESULTS

A variety of lesions, including interstitial inflammation and fibrosis, tubular degeneration, hyperplasia and tubular dilation, were observed in the renal tissue of both NTA-treated and control animals. The frequency with which some of these lesions were observed at various times during the study in each treatment group are noted in Table 1. The lesions listed in this table represent only the most frequently observed and characteristic alterations; the table does not include a complete catalogue of every lesion seen.

In general, the effects of NTA were most pronounced in the epithelium of both the renal pelvis and the PCTs. Changes in the renal pelvic transitional epithelium were first observed in animals that had been given 7·3 mmol NTA/kg for 13 days (Table 1). The development of focal haemorrhage, necrosis, erosion, and hyperplasia of the epithelium of the renal pelvis (Fig. 1) were the most prominent lesions noted in this dose group over the whole period of the study. Such changes were not observed in any of the rats given the lower dose of NTA or in the controls (Table 1).

The most characteristic NTA-associated change in the structure of the renal cortical tissue was the development of cytoplasmic vacuolation in the PCT epithelium. Based on the relative extent of cytoplasmic involvement, three grades of vacuolation were distinguished in the NTA-treated rats. Cellular swelling and almost complete displacement of the cytoplasm by vacuoles characterized the most severe (Grade 3) level of cytoplasmic vacuolation observed (Fig. 2). Tubular cells were classified at the intermediate (Grade 2) level of vacuolation if they were less swollen and had generally smaller vacuoles than the most severely affected cells (Fig. 3). In the least severe form of vacuolation observed (Grade 1), the cells were not swollen and contained only a small number of vacuoles, which were usually located at about the same level as the nucleus (Fig. 4). Even this minimal level of treatment-related vacuolation could be readily distinguished from the small nontreatment-related vacuoles that lined the area adjacent to the brush border of PCT cells in both control and NTA-treated rats (Fig. 5). The incidence of tubules with this type of 'brush border' vacuolation is not included in Table 1.

The observed frequency of PCT cell cytoplasmic vacuolation was much greater in the rats given 7·3 mmol NTA/kg than in those given the lower dose (Table 1). However, within these groups the response of the individual animals to treatment with NTA varied widely. The number of vacuolated tubules observed in rats given NTA at either dose level did not increase from day 13 until the end of the study. Small groups of atrophic and hyperplastic tubules were observed in control as well as treated rats. As noted in Table 1 these tubules were placed in one of three categories (basophilic tubules, simple tubular hyperplasia, tubular nodular hyperplasia) on the basis of the relative change in tubule cell size and number and the appearance of the epithelial cell cytoplasm.

Basophilic tubules included a variety of tubular changes that ranged from atrophy to mild hyperplasia. The most prominent characteristics of these tubules were increased cytoplasmic basophilia, a relative increase in cell number and decreased or unchanged tubule diameter (Fig. 6). Compared to the animals in the control group, there was an increase of about 50% in the total number of basophilic tubules that were observed in the rats given either of the doses of NTA during the course of the study (Table 1).

Tubules with a marked increase in cell number and little or no increase in tubule diameter were considered to show simple tubular hyperplasia (Fig. 7). These tubules were further subclassified on the basis of whether the epithelial cells were vacuolated, basophilic or eosinophilic. Like the basophilic tubules, simple tubular hyperplasia was found to occur in both control and NTA-treated rats. However, the frequency with which this hyperplastic alteration was observed increased in the NTA-treated rats in a dose-dependent manner. This increase was evident in all three subcategories of simple tubular hyperplasia. However, if the two groups of NTA-treated rats are considered together, approximately 40% of the increase in simple tubular hyperplasia was of the vacuolated cell type. This vacuolated hyperplastic lesion was not found in tissue from control animals and was observed approximately ten times more often in the 7·3-mmol/kg group than in the 0·73-mmol/kg group (Table 1).

Except for one isolated case, the development of tubular nodular hyperplasia was observed only in the kidneys of rats given the higher dose of NTA. The tubules in this category were more than twice the diameter of adjacent uninvolved tubules, contained 20 or more cells in a cross-section, and frequently had markedly swollen or hypertrophic epithelial cells

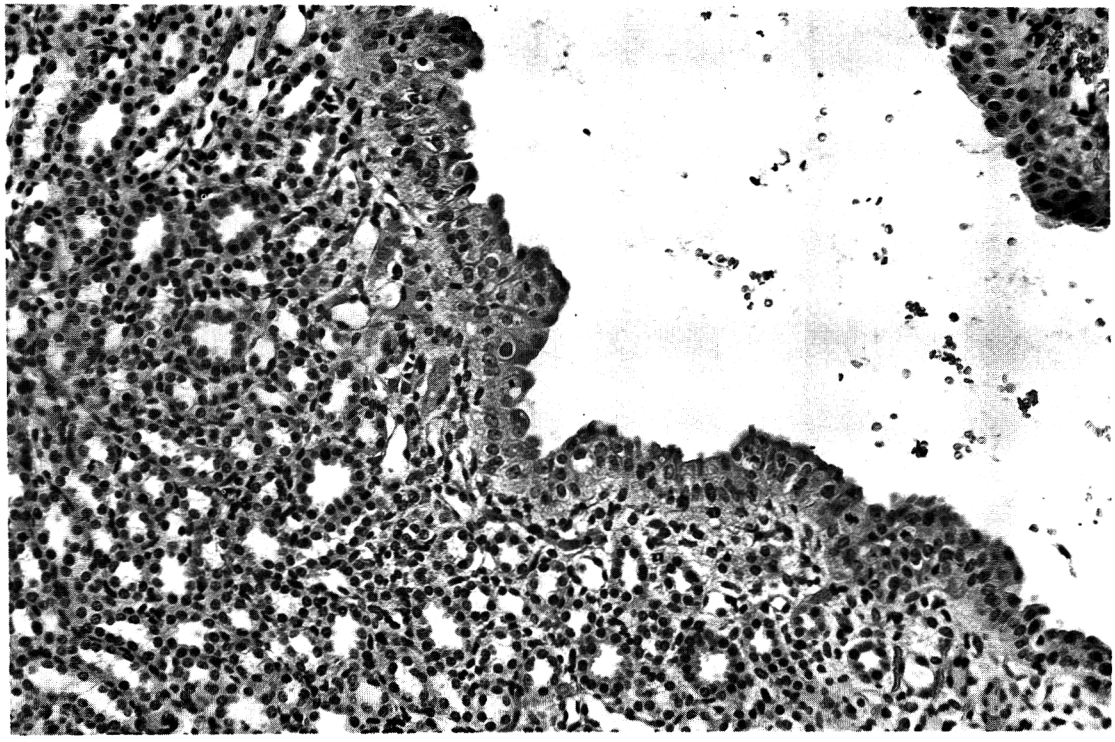

Fig. 1. Marked hyperplasia and sloughing of the transitional epithelium of the renal pelvis of a rat given by gavage 7·3 mmol NTA/kg body weight/day for 23 days. Haematoxylin and eosin (H & E) × 100.

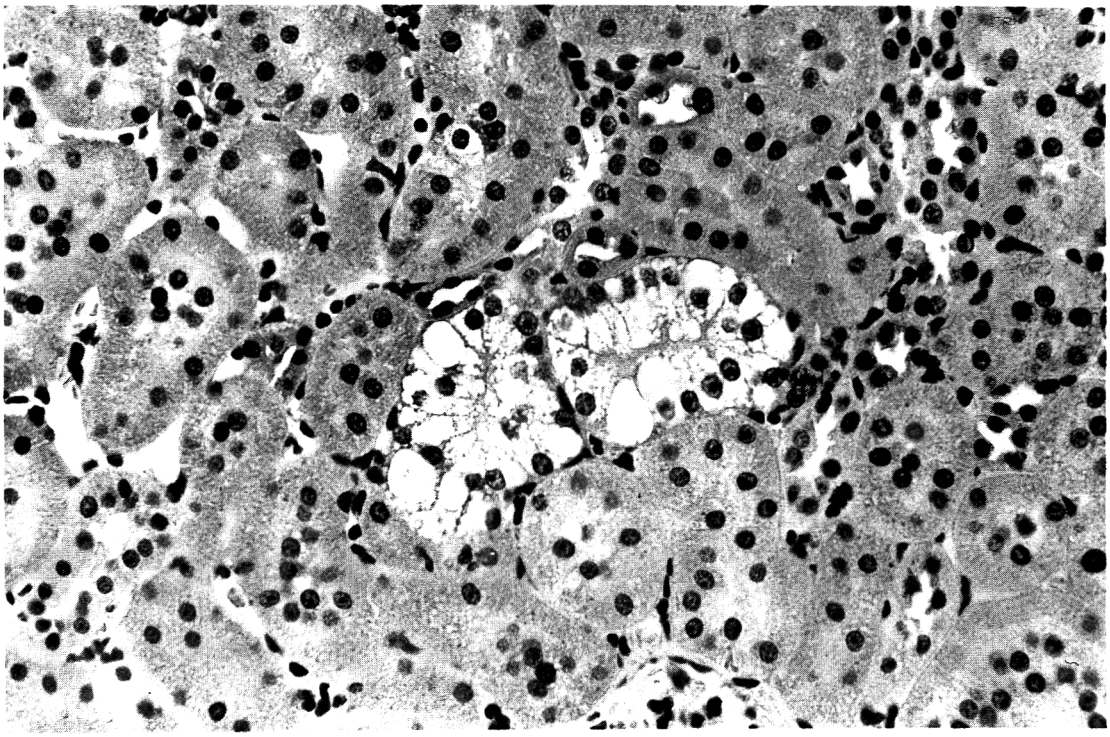

Fig. 2. Severe (Grade 3) cytoplasmic vacuolation of two proximal convoluted tubules observed in a rat given by gavage 7·3 mmol NTA/kg body weight/day for 23 days. H & E × 185.

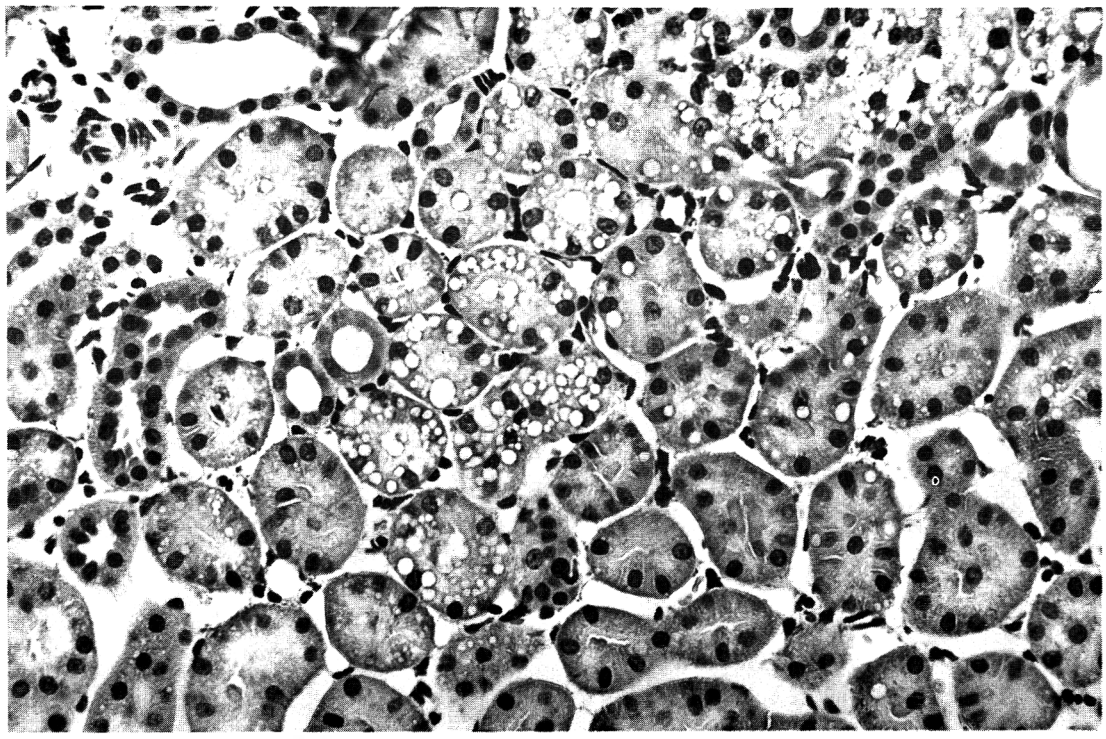

Fig. 3. Several cross-sections of proximal convoluted tubules with vacuolated epithelial cells of intermediate (Grade 2) severity in a rat given by gavage 7·3 mmol NTA/kg body weight/day for 13 days. H & E × 195.

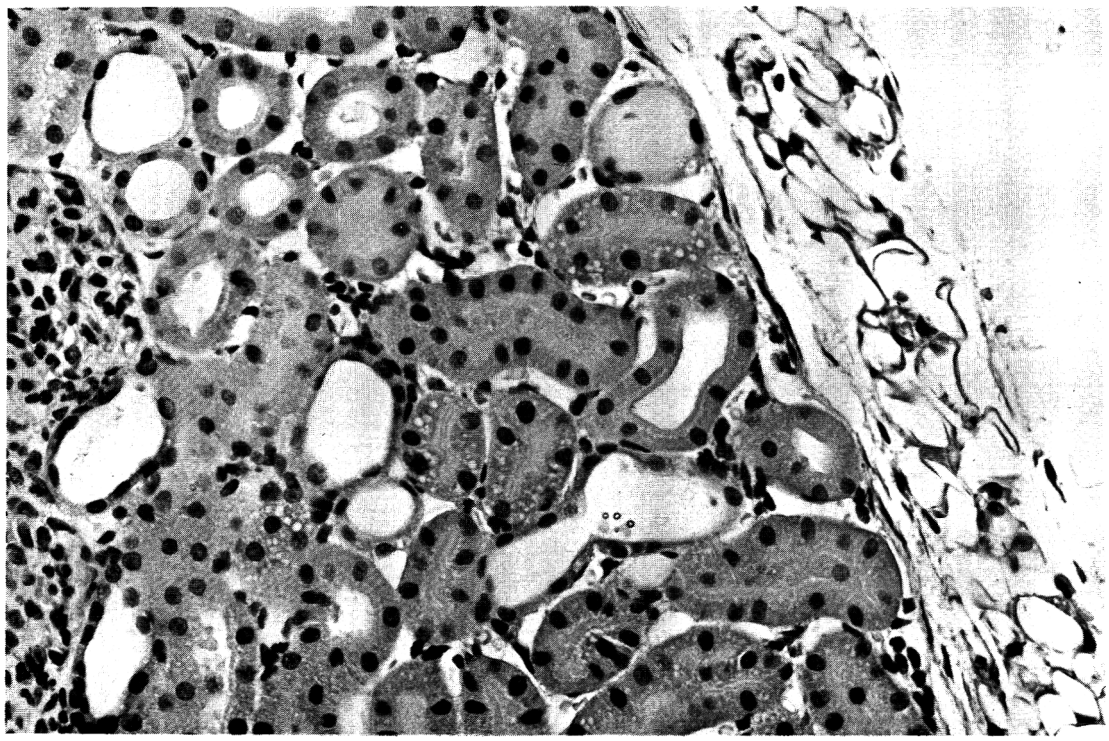

Fig. 4. An example of the relatively small vacuoles, usually located in the mid or basal portions of the proximal convoluted tubular cells, that characterized the least severe (Grade 1) level of cytoplasmic vacuolation as observed in the proximal convoluted tubular cells of a rat given by gavage 0·73 mmol NTA/kg body weight/day for 9 days. H & E × 195.

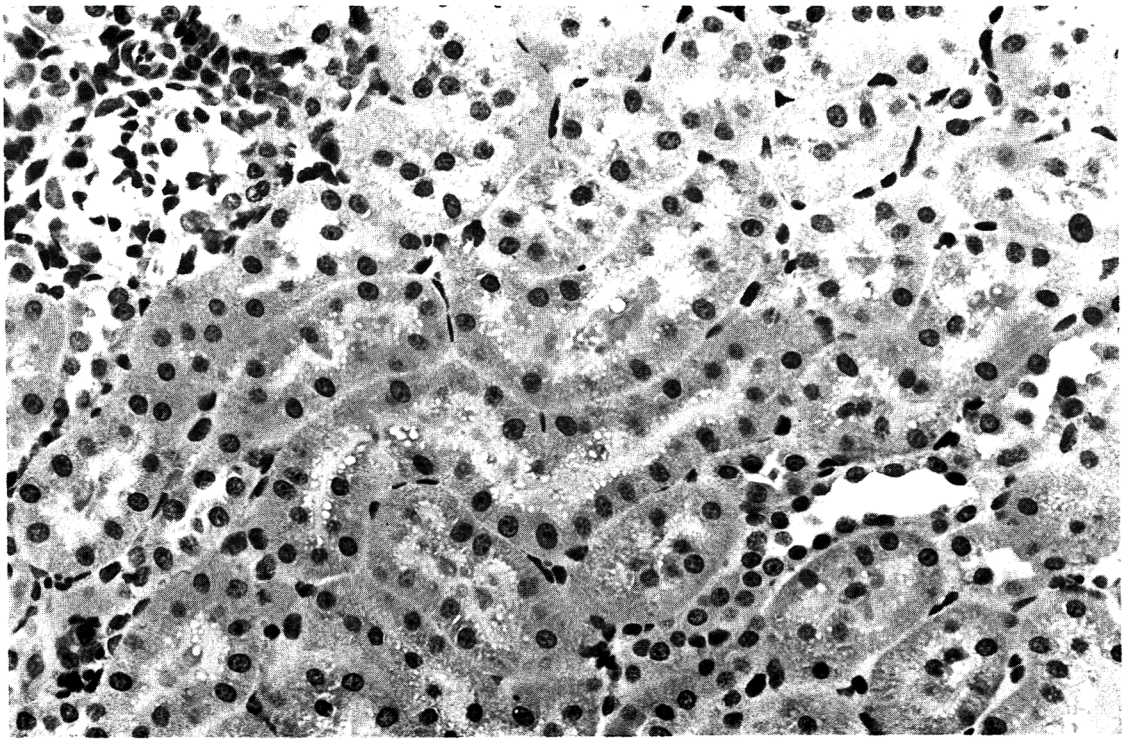

Fig. 5. Small vacuoles located near the brush border in the proximal convoluted tubule cells of a control rat. The incidence of such vacuoles was not related to NTA treatment. H & E × 200.

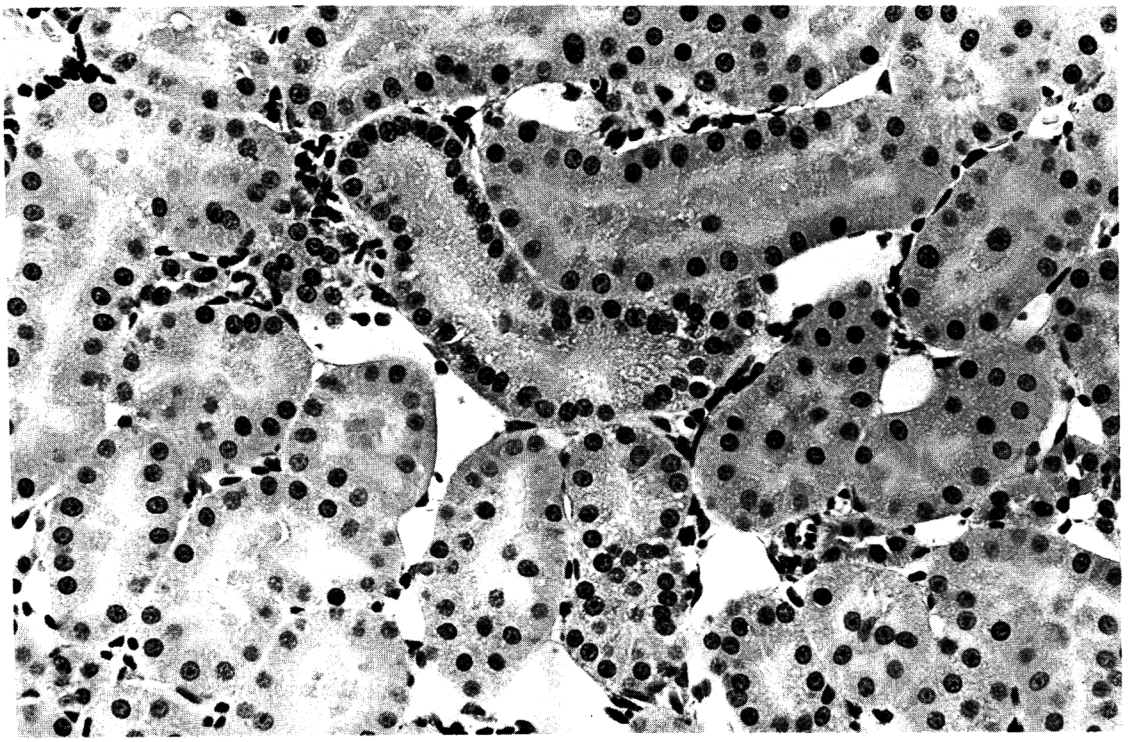

Fig. 6. Proximal convoluted tubules with a relative increase in cell number from a control rat killed after 13 days typify the appearance of tubules in the basophilic tubule category (see text). H & E × 195.

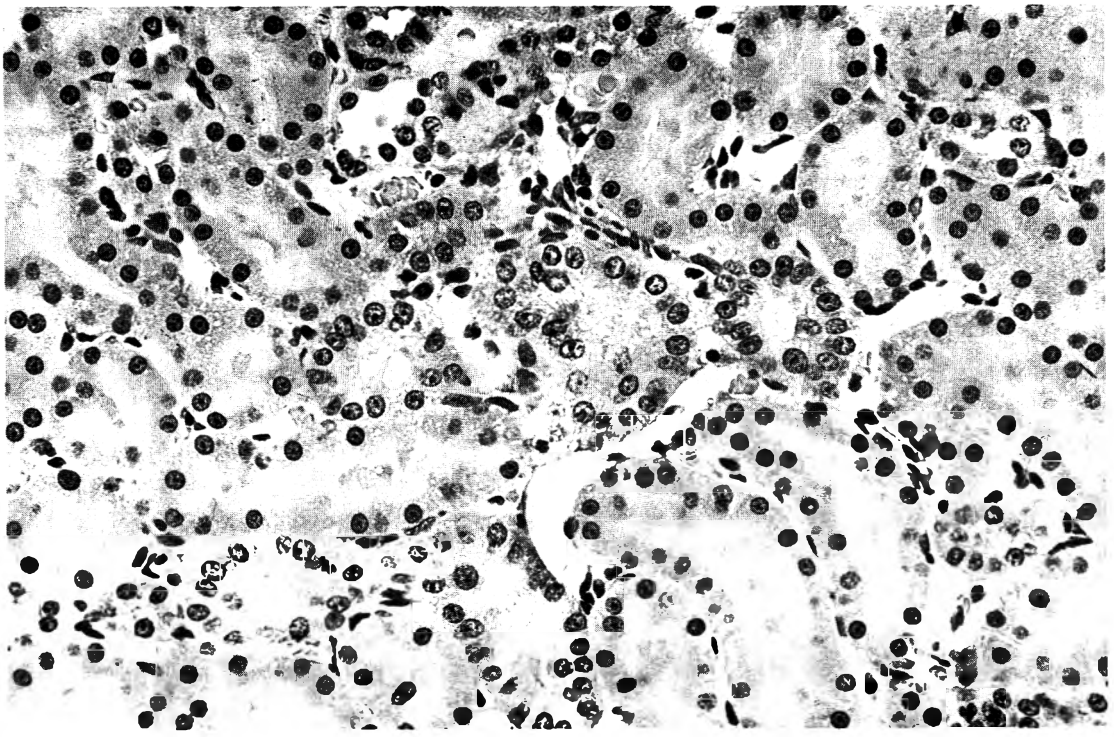

Fig. 7. Simple tubular hyperplasia of the basophilic cell type which developed in a rat given by gavage 7·3 mmol NTA/kg body weight/day for 23 days. H & E × 200.

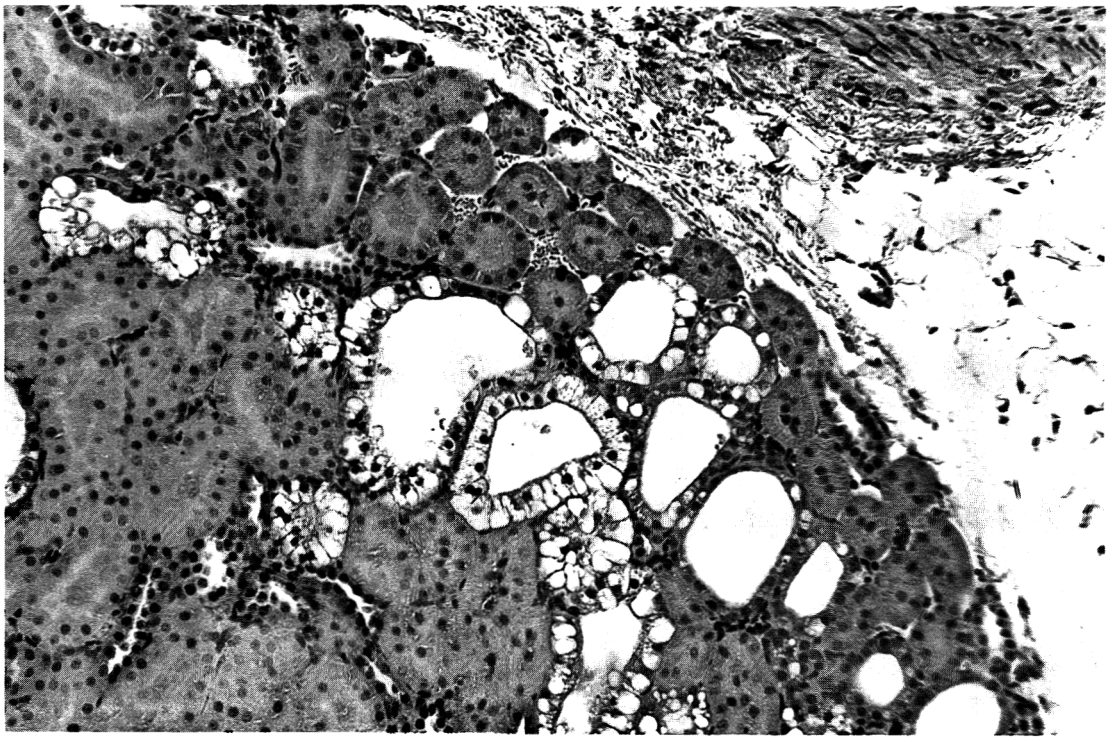

Fig. 8. Highly vacuolated cells are prominent in this focus of tubular nodular hyperplasia that developed in a rat given 7·3 mmol NTA/kg body weight/day for 20 days. H & E × 100.

Table 1. *Incidence of renal lesions in rats given by gavage 0·73 or 7·3 mmol $Na_3NTA.H_2O$/kg body weight/day for up to 30 days*

Type of renal lesion	NTA dose (mmol/kg body weight/day)	Combined no. of lesions observed in kidney sections from two rats killed on day							
		9	13	16	20	23	27	30	Total
Leucocytic accumulation*	0	31	4	38	8	10	1	7	99
	0·73	26	8	10	0	11	2	16	73
	7·3	29	10	9	3	6	16	11	84
Fibrosis*	0	26	5	5	10	1	1	15	63
	0·73	24	7	6	0	6	5	13	61
	7·3	12	15	13	2	15	24	16	97
Tubular vacuolation†:									
Grade 1 (mild)	0	0	0	0	0	0	0	0	0
	0·73	0	0	0	0	0	1	1	2
	7·3	7	71	159	15	8	40	26	326
Grade 2 (moderate)	0	0	0	0	0	0	0	0	0
	0·73	24	7	12	1	0	10	3	57
	7·3	18	74	184	5	8	>200	8	>497
Grade 3 (severe)	0	0	0	0	0	0	0	0	0
	0·73	0	0	0	0	0	0	0	0
	7·3	20	61	57	24	35	108	9	314
Basophilic tubules†	0	58	30	49	47	12	10	15	221
	0·73	61	39	41	13	54	124	43	375
	7·3	44	114	15	10	52	42	33	310
Simple tubular hyperplasia‡:									
Vacuolated	0	0	0	0	0	0	0	0	0
	0·73	0	0	0	10	0	8	0	18
	7·3	0	53	23	48	26	23	32	205
Basophilic	0	2	0	12	16	3	0	0	33
	0·73	0	3	10	11	5	22	2	53
	7·3	0	52	11	17	26	9	16	131
Eosinophilic	0	0	0	0	0	0	0	0	0
	0·73	0	0	1	0	0	0	1	2
	7·3	0	139	0	0	0	0	0	139
Tubular nodular hyperplasia†									
Vacuolated	0	0	0	0	0	0	0	0	0
	0·73	0	0	0	0	0	0	0	0
	7·3	0	0	0	12	4	3	12	31
Basophilic	0	0	0	0	0	0	0	0	0
	0·73	0	0	0	0	0	0	1	1
	7·3	0	0	0	2	0	1	0	3
Pelvic epithelial lesions†:									
Hyperplasia	0	0	0	0	0	0	0	0	0
	0·73	0	0	0	0	0	0	0	0
	7·3	0	3	2	7	1	2	1	16
Sloughing	0	0	0	0	0	0	0	0	0
	0·73	0	0	0	0	0	0	0	0
	7·3	0	0	1	5	5	6	3	20
Erosion	0	0	0	0	0	0	0	0	0
	0·73	0	0	0	0	0	0	0	0
	7·3	0	0	1	0	1	1	1	4

*Values indicate the number of sites showing the lesion.
†Values indicate the number of affected tubule cross-sections observed.

(Fig. 8). In addition, these tubules frequently had two or more layers of epithelial cells. Although sometimes cells appeared to fill the lumen of these tubules, in most instances the lumen was either of normal size or dilated. The tubular nodular hyperplastic lesions were subclassified as vacuolated or basophilic according to the appearance of their epithelial cell cytoplasm.

DISCUSSION

It was previously demonstrated that cytoplasmic vacuolation develops in PCT cells within a few hours after a single gavage dose of NTA at levels of 0·073 mmol/kg or more and that the severity of this lesion is dose related (Merski, 1981). The results of the present study confirm the dose dependency of the vacuolar lesion. This simple dose-response relationship is complicated somewhat by the fact that the number of vacuolated tubules observed did not increase appreciably with the length of NTA treatment. Variations in the response of the individual animals to NTA treatment and the small number of rats treated may account for the lack of a more clearly defined effect of length of exposure. Other factors may

also be involved. For example, the tendency of the tubules in each kidney to develop vacuolation may vary so that only a given percentage of renal tubules will be affected at a particular dose of NTA. Since the renal tubular population is heterogeneous, a functional difference between tubules expressed as a variation in their sensitivity to NTA-associated vacuolation seems plausible. The dynamics of the vacuolated tubule population could provide the basis for another means of explaining the lack of NTA action over time: as some tubules become vacuolated other previously vacuolated tubules become hyperplastic or atrophic. This process could result in a relatively stable population of vacuolated tubules.

The results of the present study also extend previous observations by demonstrating that the vacuolar lesion of the PCTs is the only alteration to develop in the kidneys of rats given 0·73 or 7·3 mmol NTA/kg body weight/day for up to 9 days. This initial change is followed in due course by the development of an increased incidence of various other lesions, most notably hyperplasia of the PCT epithelium.

Treatment with NTA resulted in an increase in the observed incidence of two classes of hyperplastic tubules. In both cases, tubules with vacuolated epithelial cells accounted for a major portion of these increases; vacuolated cells were present in 30 and 70% of the tubules showing simple hyperplasia in rats treated with 0·73 or 7·3 mmol NTA/kg, respectively (Table 1). In addition, vacuolated epithelial cells were found in over 90% of the tubules with nodular hyperplastic lesions. These data not only demonstrate a close association between vacuolation and hyperplasia but also strongly suggest that the structural alterations that develop in the renal tubules during NTA administration occur in a sequential pattern beginning with cytoplasmic vacuolation and proceeding to increasingly more severe forms of hyperplasia. It is also evident that both the number of tubules affected and the rate at which these alterations progress increases in a dose-related fashion.

Previous work has demonstrated that ingestion of NTA by rats and mice at levels of approximately 0·03% (*c.* 0·073 mmol/kg/day) or less will not induce either cytoplasmic vacuolation, tumour formation or other forms of urinary tract toxicity (Lijinsky *et al.* 1973; Merski, 1981; National Cancer Institute, 1977; Nixon, 1971; Nixon *et al.* 1972). However, these same studies, as well as more recent work show that consumption of a diet containing NTA at a level of 0·15% (*c.* 0·55 mmol/kg/day) or more induces both pathological changes (C.L. Alden, personal communication, 1981) and a disruption of the normal physiology of the urinary tract (Anderson *et al.* 1982; Anderson & Kanerva, 1978a). The results of the present study are in agreement with these data and, in addition, clearly demonstrate a relationship between the acute vacuolation and the chronic proliferative lesions that occur in the PCTs when NTA is administered at a high dose level. Other studies conducted at our laboratory deal with the relationship between these chronic proliferative lesions and tumour formation, and the role of cation metabolism in lesion formation (Anderson *et al.* 1982; Anderson & Kanerva, 1978a,b). However, further work must be done before the role these lesions play in the development of NTA-associated tumour formation can be completely understood.

Acknowledgement—The author wishes to thank J. I. Poynter for his assistance with the in-life portion of this study.

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Fd Chem. Toxic. Vol. 20, pp. 441 to 450, 1982
Printed in Great Britain.

0278-6915/82/040441-08$03.00/0

THE PATHOGENESIS OF RENAL CORTICAL TUMOURS IN RATS FED 2% TRISODIUM NITRILOTRIACETATE MONOHYDRATE

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(*Received 12 November 1981; revision received 20 January 1982*)

Abstract—To help assess the relationship between the renal toxicity and tumorigenicity associated with the administration of high doses of nitrilotriacetate (NTA) we have reviewed slides of sections from the kidneys of rats used in the National Cancer Institute bioassay of NTA. Trisodium NTA fed to Fischer 344 rats at 2% in the diet for 2 yr exerts a persistent toxic effect on the renal cortex which is manifested morphologically as vacuolation of proximal convoluted tubular epithelium and exacerbation of age-related nephrosis. Additionally, two pathogenic pathways leading to tumour formation in NTA-treated rats are suggested. A specific pathway initiated by vacuolation of proximal convoluted tubular epithelium leads to hyperplasia; reasons are advanced for the view that hyperplasia progresses to neoplasia. A possible concomitant pathway, which we suggest is nonspecific, is associated with regenerative proliferation in kidneys affected by severe age-related nephrosis. These data support the concept that there is a causal relationship between NTA-associated tubular toxicity and tumorigenicity. Doses of NTA that do not induce toxicity do not induce tubular tumours as demonstrated in this and in two other major long-term studies. Hence studies to investigate the pathogenesis of the toxic response provide an insight that suggests the basis for the development of NTA-associated tumours.

INTRODUCTION

Nitrilotriacetic acid (NTA) efficiently sequesters divalent cations. NTA has been used in Canada for 10 years as a detergent builder, and such use may attain comparable levels in the United States. The U.S. National Cancer Institute (NCI) has demonstrated that NTA administered to rats at high doses for 18 months or 2 years is associated with tumorigenicity in, and restricted to, the urinary tract (NCI, 1977). To help assess the significance of the NCI NTA bioassay results, we are attempting to establish the pathogenesis of tumour formation. In short-term pathogenetic studies at dose levels comparable to those used in the NCI bioassay we have previously demonstrated significant urinary tract toxicity in rats fed NTA (Alden, Kanerva, Anderson & Adkins, 1981). Since the goal of the NCI bioassay was to demonstrate tumours rather than toxic effects, the NCI report did not specifically address toxic injury to the urinary tract. Hence, to help assess the relationship between the toxic and tumorigenic response we have obtained from the NCI Archives and reviewed the slides of urinary tracts from NTA-fed rats, and we now report the results of that review. Attention will be restricted to the male rat renal cortex in this report since the incidence of tumours of tubular origin was higher in males exposed to NTA.

EXPERIMENTAL

All available slides from male Fischer 344 rats fed trisodium nitrilotriacetate monohydrate ($Na_3NTA.H_2O$) at a dietary level of 2% for 2 yr and corresponding controls were retrieved from the NCI Archives maintained by Tracor Jitco at Rockville, MD. Additionally, slides from ten male animals in the mid- and low-dose groups (given 0·2 and 0·02% $Na_3NTA.H_2O$, respectively) and from ten animals in the control group were selected randomly using a random number table. One transverse section of kidney from each rat was selected randomly for detailed examination. Selected slides of kidney sections from all groups were mixed and examined on a blind basis.

Abbreviations: NCI = National Cancer Institute; NTA = trisodium nitrilotriacetate monohydrate; PCT = proximal convoluted tubule.

RESULTS

Histomorphological alterations in the renal cortices of the rats selected for examination included:

(i) cytoplasmic vacuolation of proximal convoluted tubular (PCT) epithelium;
(ii) lesions characteristic of age-related nephrosis (progressive glomerulonephritis; Figs 1, 2 & 3);
(iii) PCT hyperplasia;
(iv) tubular neoplasia.

Cytoplasmic vacuolation of PCT epithelium occurred in every kidney examined from rats in the high-dose group (2% NTA in the diet; Table 1). Similar vacuolation did not occur in the kidneys from male rats in the control, low-dose or mid-dose groups. The severity of the lesion varied from minimal focal to severe diffuse (Fig. 4) PCT cell cytoplasmic vacuolation. Rats with severe age-related nephrotic lesions in which there was a minimum of functional parenchyma were still affected by PCT epithelial cell cytoplasmic vacuolation in the occasional residual functional nephron (Fig. 5).

Age-related nephrosis was recognized by any com-

Table 1. *Incidence of renal cortical lesions in Fischer 344 male rats fed diets containing 0, 0·02, 0·2 or 2% $Na_3NTA.H_2O$ for 2 yr*

Parameter	Incidence* in rats given $Na_3NTA.H_2O$ at a dietary level (%) of			
	0 (Control)	0·02	0·2	2
PCT cell cytoplasmic vacuolation	0/10	0/9	0/10	23/23
Age-related nephrosis†	10/10 (1·4)	9/9 (1·9)	10/10 (1·7)	23/23 (3·9)
PCT cell hyperplasia	5/10	4/9	5/10	23/23
Renal tubular neoplasia	0/10	0/9	0/10	4/23

*No. of slides showing lesion/no. of slides examined. One slide was evaluated per rat. The quality of the kidney section from the tenth rat in the 0·02% dose group was inadequate for evaluation.

†The mean grade of severity is given in brackets. Age-related nephrosis was graded on the basis 1 = minimal, 2 = mild, 3 = moderate, 4 = severe.

bination of the following inter-related cortical alterations:

(i) tubular dilation;
(ii) tubular colloid casts;
(iii) endogenous tubular cytoplasmic pigmentation;
(iv) granular degeneration of PCT epithelium;
(v) nephron atrophy (PCT cytoplasmic basophilia and PCT basement membrane thickening);
(vi) interstitial mononuclear cell infiltration and/or fibrosis;
(vii) glomerular alterations (fibrosis or hyalinization of Bowman's membrane and/or the glomerular tuft);
(viii) PCT cell cytoplasmic foaminess primarily occurring in tubules near the corticomedullary junction.

The mean grade of severity of the lesions associated with age-related nephrosis is indicated in Table 1. The typical high-dose male rat was severely affected by age-related nephrosis while mid- and low-dose male rats were affected to a degree similar to that of the controls.

PCT hyperplasia was significantly increased in the kidneys of the male rats given the high dose of NTA in comparison with the controls. The incidence and type of PCT hyperplasia in the low- and mid-dose group rats were similar to those in the controls. Hyperplastic PCT changes were put into subcategories on the basis of whether the cytoplasm was eosinophilic, basophilic or vacuolated. Also, subcategories were based upon growth characteristics including the following:

(i) Simple tubular hyperplasia (Fig. 6): tubules so designated were increased in cross-sectional diameter because of an increase in component cell size and number.
(ii) Tubular hyperplastic nodules (Fig. 7): the diameters of the cross-sections of such lesions were equal to or slightly greater than those of the glomeruli. The nodules were solid or cystic, but when cystic were lined by cells of increased size that were usually multilayered.
(iii) Adenomatous hyperplasia (Fig. 8): foci of adenomatous hyperplasia were often readily delineated from surrounding parenchyma; often a single focus consisted of multiple solid or cystic multilayered nodules. The component cellular nodules often elicited a response in the basement membrane or interstitium creating an impression of encapsulation. Cells in these foci were hyperchromatic in comparison with the cells in the simple and nodular hyperplastic categories. A key feature in this category was the appearance of growth no longer restricted by the parent nephron.

The incidence of PCT cell hyperplasia in the control and treated groups is shown in Table 1. PCT cell proliferation in the control, low- and mid-dose groups was restricted to simple tubular hyperplasia of the basophilic cell type and was of similar incidence and grade in all three groups. All of the types of hyperplasia described above occurred in the high-dose NTA-treated group, and the incidences are shown in Fig. 9. Hyperplastic lesions of all growth characteristics were sometimes comprised of cells with varied rather than uniform cytoplasmic appearance. The lesions were then designated according to the predominant cell type. Transition from vacuolated to basophilic cell type occurred in foci of adenomatous hyperplasia (Fig. 8).

Renal cortical tubular tumours occurred only in the group fed 2% NTA (Fig. 10). In the 23 high-dose rats evaluated four tubular tumours occurred comprising three adenomas and one adenocarcinoma. Of the three adenomas, two were made up of eosinophilic cell types and one of basophilic cell type. The growth pattern in the adenomas was either solid or tubular. The adenocarcinoma was composed of cells with lightly basophilic cytoplasm in a tubular growth pattern.

DISCUSSION

$Na_3NTA.H_2O$ at doses comparable to those used in the NCI bioassay has been previously reported to have significant toxicological effects on the renal cortex, which are manifested morphologically as vacuolation of PCT epithelium and exacerbation of age-related nephrosis in rats (Nixon, Buehler & Niewenhuis, 1972). The results of our review of the slides from the

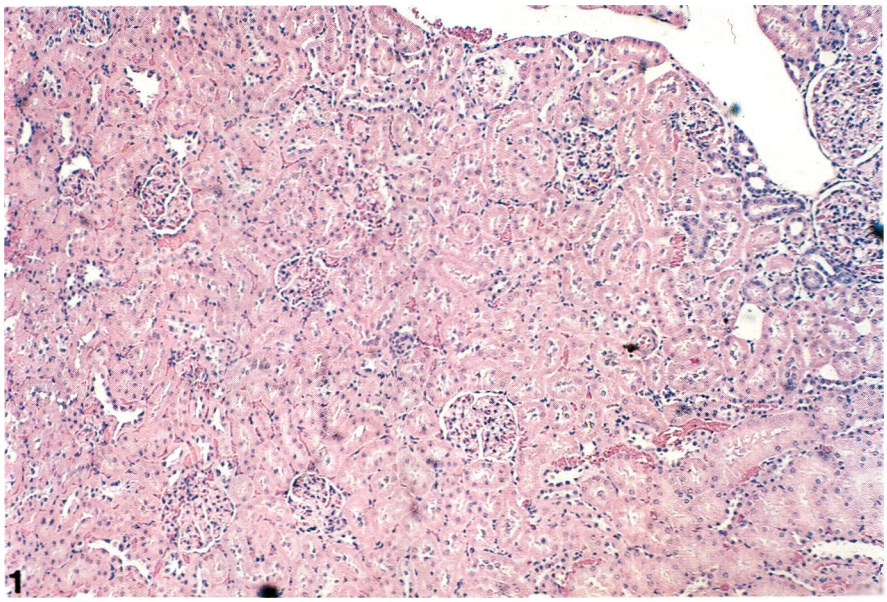

Fig. 1. Section of the renal cortex of a Fischer 344 male rat fed the control diet for 2 yr in the NCI bioassay of NTA. The section shows typical, minimal age-related nephrosis.

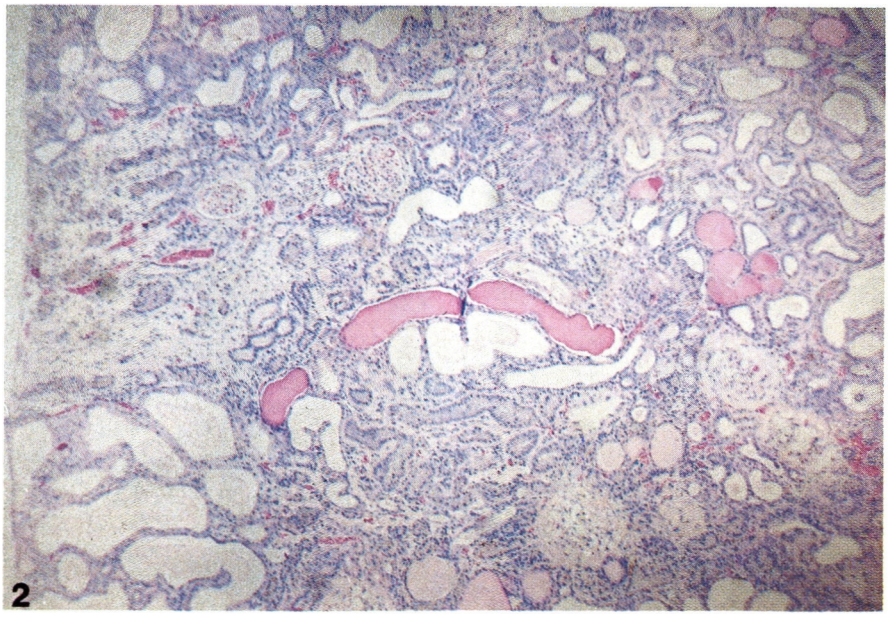

Fig. 2. Section of the renal cortex of a Fischer 344 male rat fed $Na_3NTA.H_2O$ at 2% in the diet for 2 yr in the NCI bioassay*. The section shows severe age-related nephrosis.

*These details apply to all of the following photomicrographs.

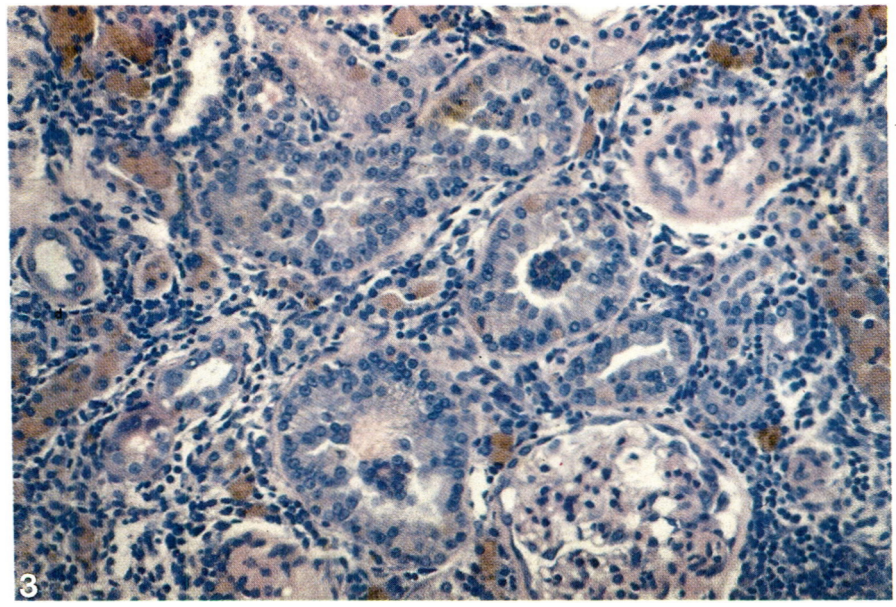

Fig. 3. Age-related nephrosis. Tubular hyperplasia of the basophilic cell type is prominent.

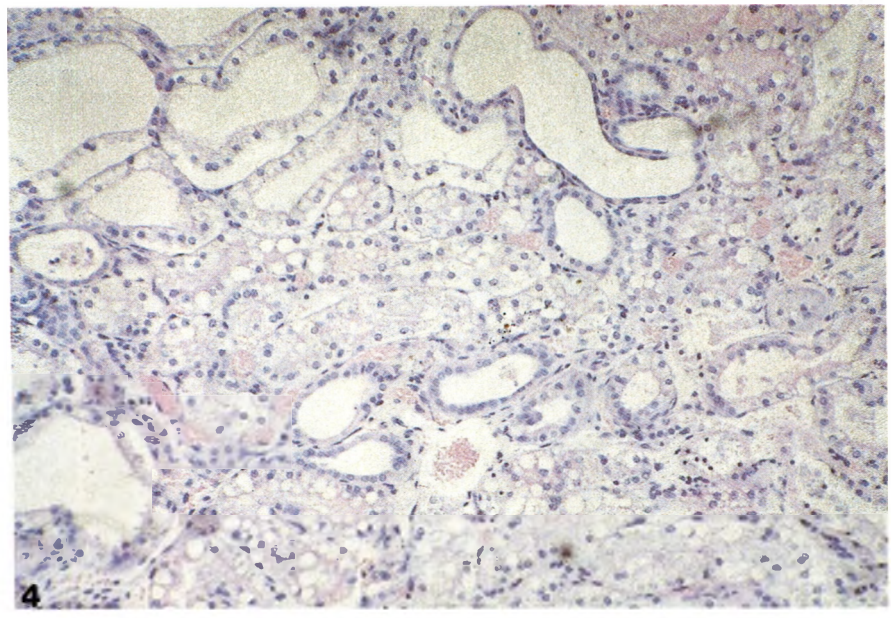

Fig. 4. Diffusely distributed vacuolation of tubular epithelium.

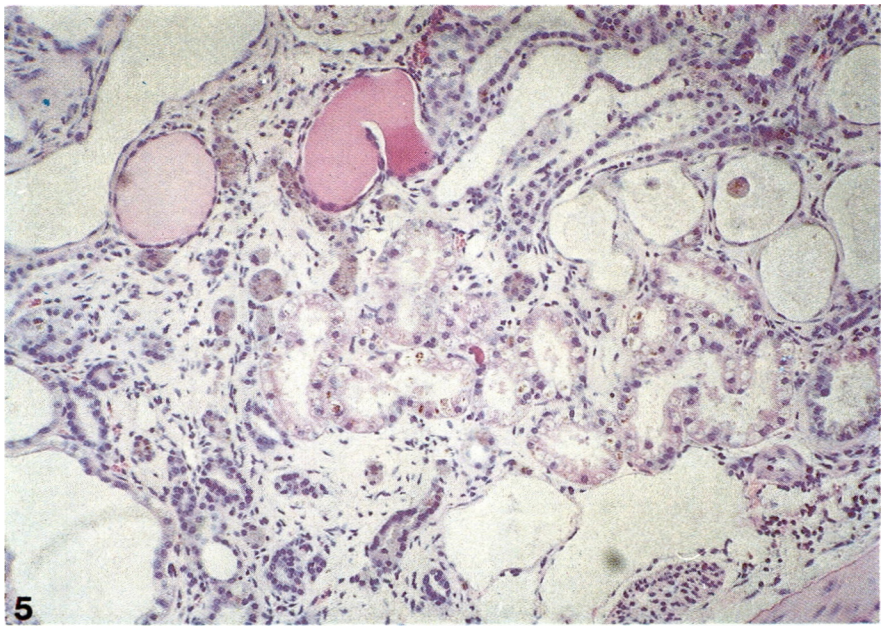

Fig. 5. Vacuolation of the tubular epithelium of a residual nephron and severe age-related nephrosis.

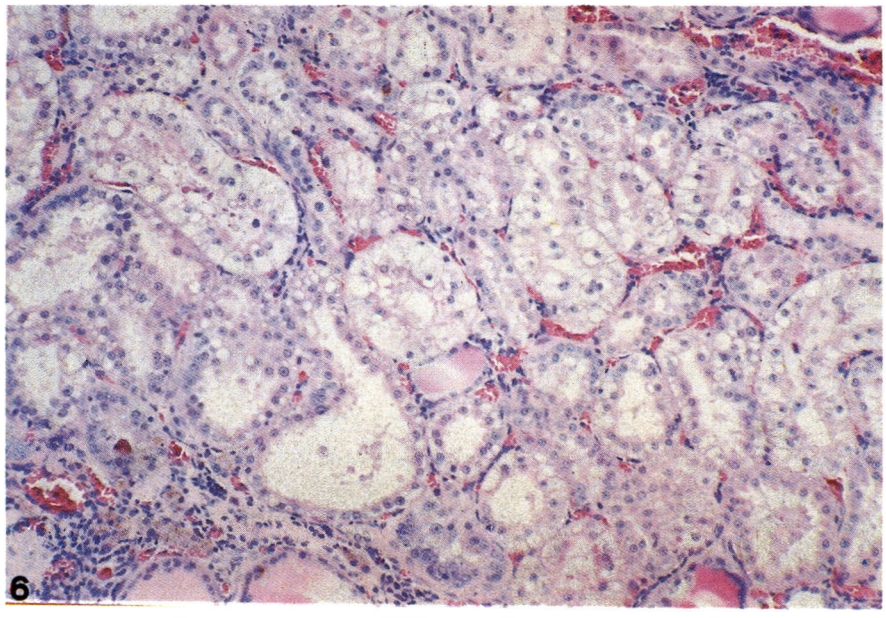

Fig. 6. Simple tubular hyperplasia of the vacuolated cell type.

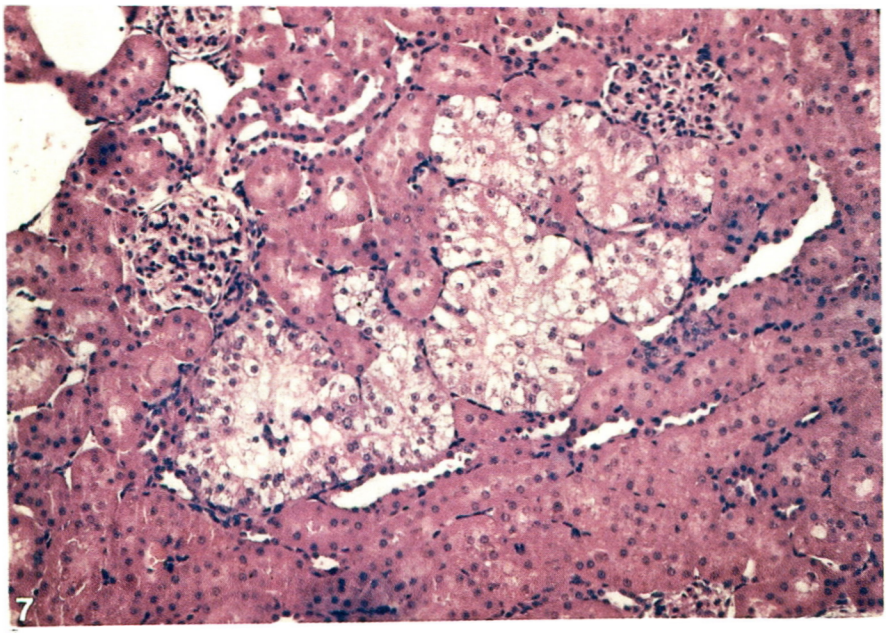

Fig. 7. Tubular nodular hyperplasia of the vacuolated cell type.

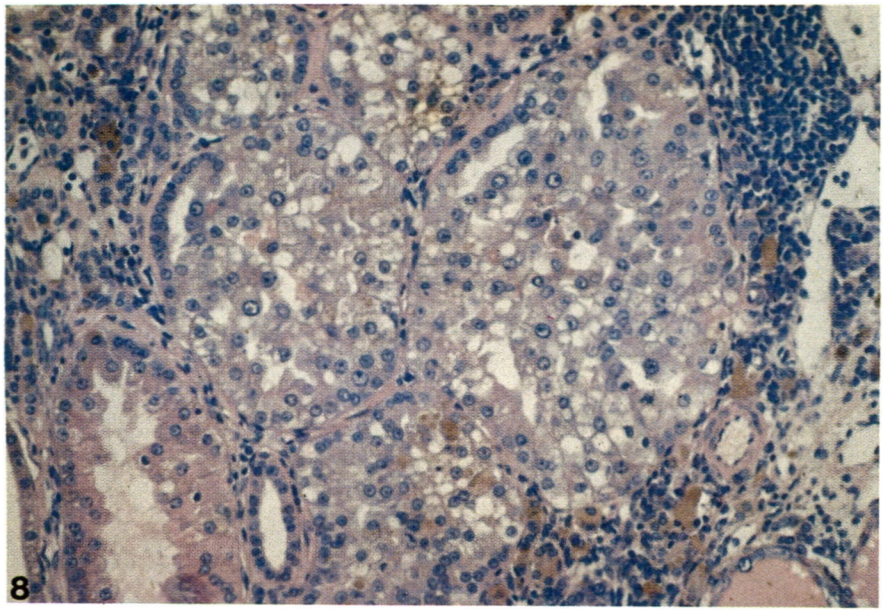

Fig. 8. A focus of adenomatous hyperplasia showing transition in cell type from a cell with vacuolated cytoplasm to a cell with basophilic cytoplasm.

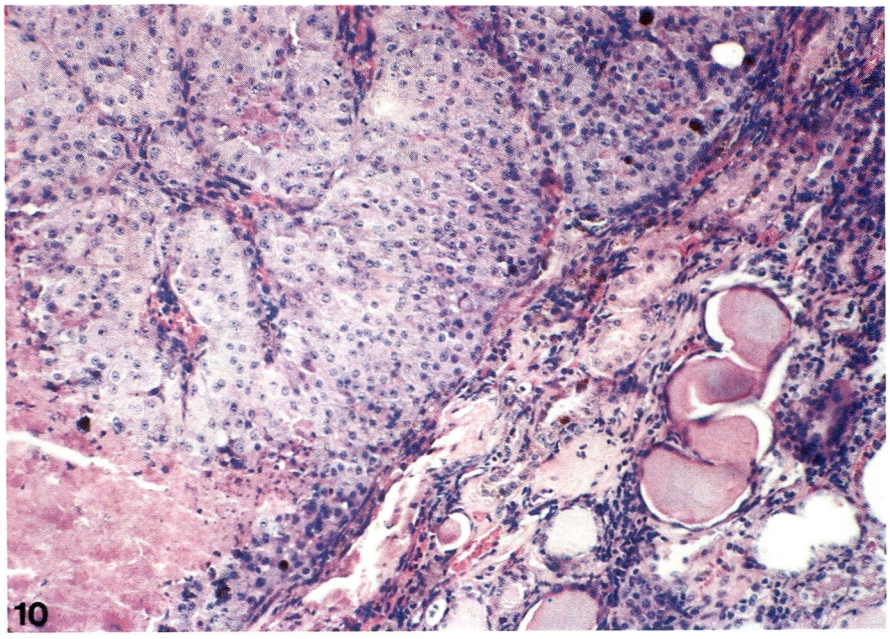

Fig. 10. Solid tubular carcinoma of basophilic cell type.

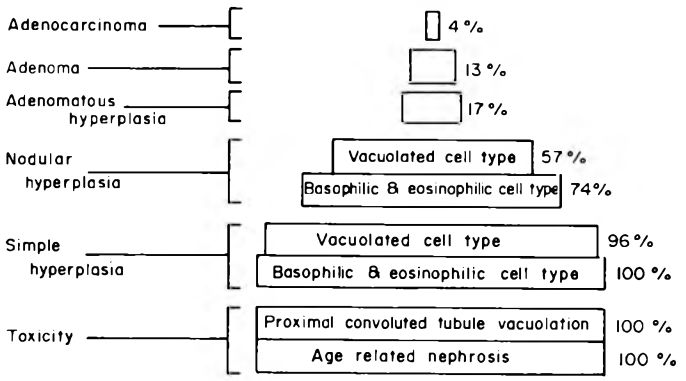

Fig. 9. The incidence of lesions in the renal cortex of Fischer 344 male rats given $Na_3NTA.H_2O$ at a dietary level of 2% for 2 yr in the NCI bioassay. The lesions are listed in ascending order on the basis of frequency and time/course sequence, with the most numerous and earliest lesions at the bottom. The pyramid thus formed has a base of lesions indicating toxicity and an apex or end point of tubular neoplasms.

NCI's bioassay clearly demonstrate the occurrence and persistence of manifestations of the nephron toxicity of NTA in rats exposed continuously for 2 yr. These effects were not reported by the NCI.

Additionally, we have previously described short-term proliferative sequelae to this toxic injury (Alden *et al.* 1981) and are now suggesting two pathways leading to tumour formation in NTA-treated rats. The first pathway (Fig. 11) is a specific pathway initiated morphologically by vacuolation of PCT epithelium, which represents swelling of the endocytotic lysosomal system of the cell (J. Merski, personal communication, 1981). Hyperplasia of the vacuolated epithelium in affected nephrons ensues. The results of this study suggest that occasional nephrons pass through successive exaggerations of the hyperplastic response which eventually result in autonomous growth. Near or at the time of transformation to autonomous growth there is apparently transition from a vacuolated to a nonvacuolated proliferative cell presumably reflecting altered exposure or response to NTA; hence, the majority of induced tumours are of the nonvacuolated cell type.

A possible concomitant pathway to tumour formation is associated with a proliferative response in kidneys affected by severe age-related nephrosis (Fig. 11). The association between age-related nephrosis and neoplasia of tubular origin cannot be demonstrated within the confines of the NCI bioassay of NTA since the control rats were only mildly affected by age-related nephrosis. However, this association is supported by the veterinary literature. An increased incidence of renal cortical tumours associated with ageing has been reported: if normal rats of strains with a high incidence of age-related nephrosis are allowed to live until spontaneous death occurs, there is a significant increase in the incidence of renal cortical tumours (Cohen, Anver, Ringler & Adelman, 1978). The positive correlation between tubular hyperplasia and age-related nephrosis in the rat is also well established (Bras & Ross, 1964). Theoretically, the tubular (regenerative) hyperplastic response should increase the chance for expression of a spontaneous mutant clone, reflected in an increased incidence of neoplasia. Age-related nephrosis of rats can be exacerbated by a wide variety of stimuli (Bras & Ross, 1964). Hence, we consider this tubular hyperplastic response to be a nonspecific manifestation of nephron injury.

The relationship between the *specific* toxic manifestation of nephron injury (i.e. PCT vacuolation) and the *nonspecific* manifestation of NTA-associated nephron injury (i.e. exacerbation of age-related nephrosis) cannot be established since the cause of age-related nephrosis is unknown.

The results of our review of the bioassay slides support the occurrence of these morphologic pathways to tumour formation in NTA-treated rats (see Fig. 9). From these data we have postulated that there is a causal relationship between NTA-associated tubular toxicity and tumorigenicity. Support for this concept exists in results from short-term dose-response studies on NTA (Merski, 1981 & 1982) and from this NCI bioassay, as well as from two other long-term NTA studies which demonstrate that doses of NTA associated with tubular tumorigenesis also induce tubular toxicity (Goyer, Falk, Hogan *et al.* 1981; Nixon *et al.* 1972). Correspondingly, doses that do not induce tubular toxicity are not associated with the development of tubular neoplasia.

The importance of this key concept becomes apparent if we consider well documented biological properties and biochemical effects of NTA. NTA is biodegradable by bacteria in environmental waters and does not bioaccumulate. NTA is not mutagenic and is not metabolized in the mammalian system. Biochemically, tumorigenic doses of NTA induce measurable alterations in cation balance. Data suggesting that NTA exerts its toxicologic and hence (considering the key concept) tumorigenic effect through perturbation of cations at the cellular level have been reviewed recently (Anderson, Alden & Merski, 1982). If NTA exerts its toxicologic and tumorigenic effect through perturbation of cation balance rather than as a direct effect on the cellular genome then it clearly acts through epigenetic means. Doses of NTA that do not perturb this cation homeostasis would not induce the toxicologic or tumorigenic response and hence would be true 'no effect' doses. At these 'no effect' doses

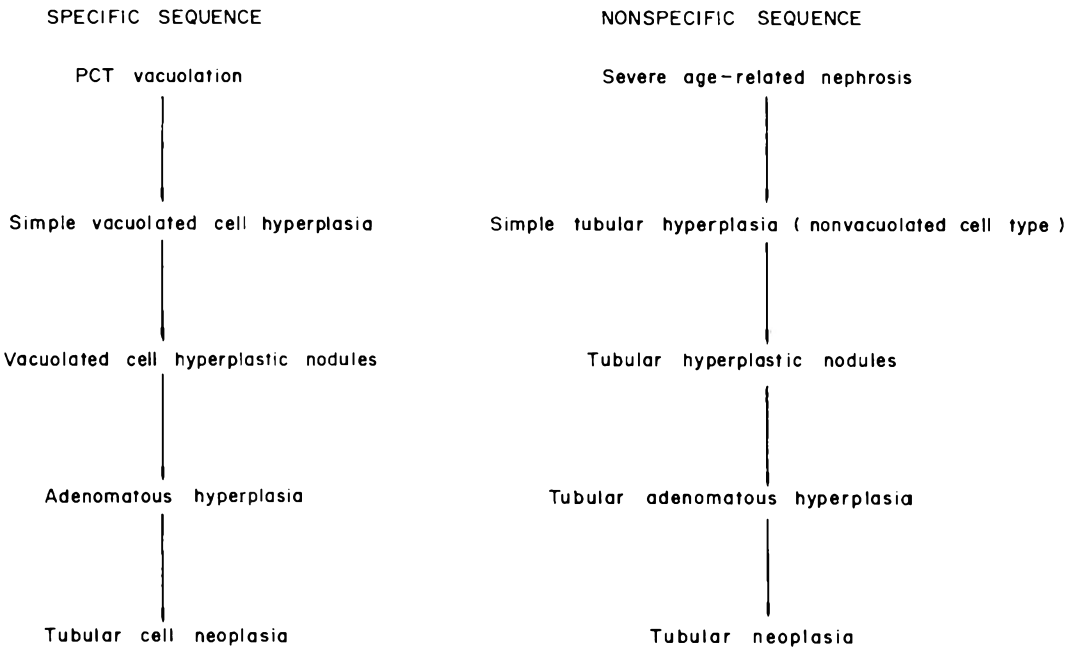

Fig. 11. Suggested sequence of events in the proximal convoluted tubules of Fischer 344 rats fed $Na_3NTA.H_2O$ at a dietary level of 2% for 2 yr.

NTA would not be a carcinogen, which is in contrast to the concept of a carcinogen diluted to undetectable levels. Short-term dose-response studies involving administration of NTA by gavage reveal that doses equivalent to 0·11 mmol/kg body weight or higher induce nephron toxicity while amounts equivalent to 0·073 mmol/kg or lower do not induce nephron toxicity (Merski, 1981).

To place the doses discussed in perspective, the 2% dietary dose of NTA that is carcinogenic in rats represents a 20-millionfold exaggeration over anticipated human exposure levels (which experience has shown to be a few ppb in drinking-water). The dietary no-effect level demonstrated in the NCI bioassay (0·02%) reflects a 200-thousandfold exaggeration of anticipated human exposure levels. These factors have been ascertained following a number of years of extensive environmental monitoring in Canada where NTA is widely used (Malaiyandi, Williams & O'Grady, 1979).

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Fd Chem. Toxic. Vol. 20, pp. 451 to 454, 1982
Printed in Great Britain.

0278-6915/82/040451-04$03.00/0

UNAFFECTED BLOOD BORON LEVELS IN NEWBORN INFANTS TREATED WITH A BORIC ACID OINTMENT

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(*Received 14 December 1981*)

Abstract—No rise in the boron content of the plasma of 22 newborn infants was demonstrated following repeated daily application of a water-emulsifying ointment containing the equivalent of 3% boric acid to the napkin region. The mean plasma-boron concentration fell over 5 days from a pretreatment value of 0·49 to 0·29 mg/litre, the corresponding values in ten untreated neonates being 0·62 and 0·21 mg/litre, respectively. No statistically significant differences were found. The results confirm the safety of such ointments for application to the skin, a conclusion predicted by theoretical estimates of the maximum possible boric acid absorption following application of the ointment.

INTRODUCTION

Skin-care products containing boric acid in low concentration have been used for decades to protect against skin irritation in the napkin region. They are sold in accordance with cosmetics legislation in many European countries provided their boric acid content does not exceed 3%.

Although the products now available have never been reported to cause poisoning or any other adverse reactions (*Meyler's Side Effects of Drugs*, 1980), their safety is still being disputed, as for instance in a recently published review based on papers published up to 1974 (Grosdanoff, 1979). Many of these papers, however, state the well-known toxicity of boric acid when used inappropriately, whereas information is lacking on the use of ointments with a low boric acid content. When the risk of boron poisoning by topical application is discussed, the role of the vehicle—and hence the bioavailability of the boric acid for absorption—must be taken into account, and few publications throw light on this subject. Calculations based on animal experiments suggest that the risk with such products is negligible, but clinical corroboration is scant, due in part to the lack of an accurate and sensitive analytical method for measuring boron in biological fluids.

The availability of a new specific method for the determination of boron in minute blood samples made possible the present investigation, which was carried out, with parental consent, in newborn babies, the age group most often exposed to the products in question. A preparation in general use, natusan (an anhydrous ointment containing the equivalent of 3% boric acid), was selected for investigation, because of its long-standing and uneventful use in baby care. To evaluate its safety, blood-boron levels in treated and untreated neonates were compared.

EXPERIMENTAL

Test material. Natusan baby ointment, supplied by A/S Alfred Benzon, Copenhagen, is a water-emulsifying ointment with the following percentage composition: sodium borate 0·15, boric acid 2·85, liquid paraffin 10·00, anhydrous glycerol 12·00, wool fat 20·00 and white soft paraffin 55·00. The glycerol–boric acid/sodium borate buffer constituting the disperse phase has a pH of about 5.

Methods. To 22 of the 32 healthy neonates included in the investigation, ordinary natusan treatment was given, a thin layer of ointment being applied to the napkin region at each change of napkin. The treatment was continued for the 5 days spent in the obstetrics department after birth. The remaining ten infants served as controls and were given no ointment treatment. The tubes containing ointment were weighed before and after each application. On average, a total of 3·0 g ointment was used for each infant in 4–5 days, corresponding to 90 mg boric acid or 15·7 mg boron. Table 1 includes the amount of ointment applied to each individual infant. The level of boron in the plasma was determined in capillary blood drawn from the heel shortly after birth (before treatment) and on days 3 and 5 after birth. The boron content in the blood of the control group was analysed at the same

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Table 1. *Amounts of ointment applied to neonates and boron concentrations in the mothers' milk and in the plasma of the infants*

Subject		Total amount applied on days 1–5		Boron concentration (mg litre)			
				Milk	Plasma* on day		
No.	Sex	Ointment (g)	Boron (mg)	Means for days 3 & 5	1†	3	5
				Treated group			
1	M	1·7	8·9	0·05	0·19	1·02	0·14
2	F	2·4	12·6	0·04	0·05	0·09	0·10
3	F	2·1	11·0	0·04	0·10	0·64	0·08
4	M	5·4	28·3	0·02	0·07	0·13	0·16
5	M	2·0	10·5	0·01	0·72	0·06	0·07
6	M	4·7	24·7	0·08	0·85	0·05	0·10
7	F	1·6	8·4	0·03	0·08	0·11	0·50
8	M	4·9	25·7	0·07	0·06	0·26	DM
9	M	1·9	10·0	0·06	0·07	DM	0·11
10	F	2·9	15·2	0·05	0·05	0·01	0·73
11	M	2·0	10·5	0·13	0·13	0·10	0·07
12	F	3·9	20·5	0·07	0·04	0·06	0·57
13	M	1·4	7·3	0·08	1·54	0·07	0·23
14	M	3·6	18·9	0·03	1·88	0·00	1·13
15	M	5·9	30·9	0·06	0·04	0·17	0·15
16	M	3·9	20·5	0·06	0·06	0·31	0·27
17	M	1·1	5·8	0·05	3·65	0·05	0·00
18	F	5·0	26·2	0·07	0·07	0·16	0·17
19	M	2·0	10·5	0·05	0·08	0·02	0·37
20	M	0·7	3·7	0·08	0·10	0·05	0·10
21	F	DM	DM	DM	0·57	0·63	0·85
22	F	DM	DM	DM	0·31	0·96	0·16
Mean ± 1 SD...		3·0 ± 1·6	15·5 ± 8·3	0·06 ± 0·03	0·49 ± 0·87	0·24 ± 0·31	0·29 ± 0·30
				Control group			
23	M			0·07	0·81	3·40	0·09
24	M			0·06	0·66	0·63	0·16
25	F			0·10	0·83	0·04	0·05
26	F			0·09	0·06	0·03	0·36
27	M			0·05	0·08	0·33	0·11
28	M			0·02	0·73	0·05	0·55
29	F			0·02	1·30	0·06	0·05
30	F			0·14	0·94	1·48	0·18
31	M			0·10	0·79	0·55	0·18
32	F			0·12	0·04	0·08	0·32
			Mean ± 1 SD...	0·08 ± 0·04	0·62 ± 0·43	0·67 ± 1·06	0·21 ± 0·16

DM = Data missing

*The six mean plasma concentration values showed random variation only and were not influenced to a statistically significant degree either by group or by day of sampling ($P < 0·05$ by analysis of variance).
†Before treatment.

times. In addition, a 14-month-old infant suffering from napkin dermatitis was examined before and after being treated with the ointment for 4 days.

To assess the significance of other possible sources of boron, the boron content of the mothers' milk was determined on days 3 and 5.

Analytical procedure. The determinations of boron in plasma and milk were performed by Dantest: The National Institute for Testing and Verification, Copenhagen, using atomic absorption spectrometry with electrothermal atomization. The method is specific for boron and has a detection limit of 0·01 mg/litre. A 20-μl sample is injected into a graphite tube, dried, charred and momentarily heated to 2700°C to release the boron atoms. Absorbance at 249·7 nm of emission from a hollow cathode lamp is measured in the atomic absorption spectrometer.

Each analysis was performed in duplicate or triplicate. The variance of duplicate measurements was approximately 1% of the mean, corresponding to a standard deviation of 1–10% in the range of 0·01–1·0 mg boron/litre plasma.

RESULTS

On day 1, the mean plasma-boron concentration of the test group (before treatment) showed no statistically significant difference from that of the control group, the values (±1 SD) being 0·49 ± 0·87 and 0·62 ± 0·43 respectively mg/litre. By day 5, the boron concentration had declined in both groups, to means of 0·29 ± 0·30 and 0·21 ± 0·16 mg/litre respectively, but neither the fall nor the difference between the groups was statistically significant (Table 1).

Both the inter- and intrasubject variations were considerable. Moreover day-to-day variations in the treatment group showed no correlation with the amounts of ointment applied. In this group, 95% confidence limits of the mean change in plasma-boron level from day 1 to day 3 were −0·60 and 0·10 mg/litre, and those of the change from day 1 to day 5 were −0·52 and 0·18 mg/litre. Thus, with regard to the possibility of a statistically derived type II error, it can be seen that the maximum possible increase following treatment for 4–5 days (0·18 mg/litre) was far less than the maximum mean difference of 0·43 mg/litre found between the two groups on day 3.

The infant suffering from napkin dermatitis had a plasma-boron level of 0·04 mg/litre before as well as after the ointment treatment.

In the samples of breast milk, the mean boron concentration was 0·07 mg/litre (0·01–0·23 mg/litre).

DISCUSSION

Plasma-boron levels found in these neonates did not on the whole differ much from those previously demonstrated by less specific methods. Thus Johnstone, Basila & Glaser (1955) and Fisher & Freimuth (1958), both using a colorimetric method, found levels of 0–7·8 and 0–1·25 mg/litre, respectively, in children.

Plasma-boron concentrations in neonates are likely to reflect those of their mothers, which are dependent on diet. This may explain why the levels measured immediately after birth were often higher than those recorded after 4–5 days of breast feeding. A normal diet, particularly when it includes fruits and vegetables with boron contents of about 5–40 mg/kg (Rasi, 1973), may contain appreciable amounts of boron, whereas breast milk has a very low boron content (Table 1).

On the other hand, no explanation can be offered for the wide day-to-day fluctuations seen in some of the children, e.g. subjects 3, 14, 17, 23 and 28 (Table 1), but the random occurrence of these fluctuations among both treated and control subjects rules out the ointment as a source. Moreover the plasma boron of the child suffering from napkin dermatitis was not influenced by the ointment application.

These results are not surprising in view of the minimal amount of boron applied and the modest absorption of boric acid from ointments already demonstrated in other studies (Draize & Kelley, 1959; Jensen, 1971; Nielsen, 1970; Stüttgen, Siebel & Aggerbeck, 1982). In this investigation, the largest amount of boron applied during the first 5 days of life was 30·9 mg (Table 1), of which presumably only a small fraction was absorbed. If the total amount of boron had been absorbed, the boron concentration of the blood would have been increased by 2·2 mg/litre, a value far below the possibly toxic level of 40 mg/litre indicated in forensic medicine (McBay, 1973) and amounting only to 1/40–1/80 of the stated minimal fatal concentrations of 87–175 mg/litre (Fisher & Freimuth, 1958).

The increase in concentration of 2·2 mg/litre was calculated from the equation

$$C_{plss} = \frac{R_{in}}{Cl}$$

which is an expression of the steady state plasma concentration, C_{plss}, when boron is continuously supplied at the rate of R_{in} (6·18 mg boron daily). Cl is the total body clearance, here taken to be 1·94 ml/min in a child with a body surface of 0·215 m^2 (weight 3·5 kg, height 52 cm). This Cl value is arrived at by using the boron clearance of 39·1 ml/min/1·73 m^2 found in adults (Farr & Konikowski, 1963) after intravenous injection of sodium pentaborate, and by assuming that boron (boric acid) is eliminated predominantly by renal filtration and partial reabsorption and also that the glomerular filtration rate per 1·73 m^2 surface of neonates is 20/125 of the glomerular filtration rate in adults, as judged by the mannitol clearance (Weil, 1955). An as yet unpublished pilot experiment has indicated that the boron clearance after ingestion of boric acid is probably not lower than that after intravenous injection of pentaborate (J. Aas Jansen, unpublished data, 1981).

The larger quantity of ointment presumably used on older children, and hence the possible rise in boron absorption, are more than counterbalanced by the greater than proportional increase in glomerular filtration rate (and in boron clearance) compared with the increase in body surface.

However, the prerequisites for complete absorption of the boron applied were not present in this investigation. The absorption of topically applied boric acid depends on the condition of the skin as well as on the boric acid formulation. Repeated treatment of a large area of broken skin with boric acid in a readily absorbed form, e.g. in solution or as pure boric acid powder, may result in toxic concentrations in the blood and tissues, as can be seen from reported cases of poisoning (Kliegel, 1980; Valdes-Dapena & Arey, 1962). Unbroken skin, on the other hand, admits at most only negligible amounts of boric acid from any formulation (Rasi, 1973) and ointments have been shown to be associated with very modest absorption even through broken skin. Thus, animal experiments on the absorption of boric acid from pure boric acid powder, from aqueous solution, from boroglycerine–glycerite, from 5% boric acid powder and from a 10% ointment showed that absorption was lowest from the ointment following application to damaged skin (Draize & Kelley, 1959). In another animal experiment, in which the ointment used in the present study was compared with an entirely water-based 3% boric acid preparation, only 1–3% of the boric acid contained in the ointment was absorbed through severely damaged skin, whereas up to 75% of that in the aqueous jelly was absorbed (Nielsen, 1970). These results were later reproduced in man by Stüttgen *et al.* (1982), who found no increase in boron excretion when natusan was applied to normal or diseased skin but found an increase in both blood and urine levels of boron when 3% boric acid jelly was applied to diseased skin.

Natusan ointment has also been applied to healthy infants in a controlled, double-blind trial including placebo treatment. No statistically significant difference in urinary boron excretion was found between the natusan- and placebo-treated infants over 8-day treatment periods (Jensen, 1971).

The safety of boric acid ointments is also reflected in the lack of reports of poisoning connected with

their use: no cases have been published involving ointments weaker than 10%, and of the three fatal cases involving 10% ointments, reported during the years 1896–1948 (Dopfer, 1905; Fellows, Campbell & Wadsworth, 1948; Hall, 1896), only one seems to afford sufficient evidence for the treatment to be considered responsible for the death (Dopfer, 1905).

During the long period in which natusan has been used, accidental ingestion of this ointment by young children has occurred from time to time. Even by this mode of administration no symptoms of toxicity have been observed, and no other harmful effects have ensued. The case published by Svenningsen (1966) may serve as an example.

Conclusions

Repeated topical application of the water-emulsifying ointment natusan, which contains 3% boric acid, was shown to cause no increase in the natural boric acid level in the blood of newborn infants. On the basis of a literature search and theoretical calculations, it is concluded that toxic boron concentrations could not be reached by the ordinary use of ointments containing such a low concentration of boric acid, even if the total amount of boron applied could be absorbed through the skin. Therefore, topical application of such preparations is safe, irrespective of the condition of the skin.

Acknowledgements—The authors wish to thank Karsten Schmidt, MSc, Spadille ApS, Fredensborg, for the statistical evaluation, and the Obstetrics Department YA, Rigshospitalet, for valuable assistance during this investigation.

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Fd Chem. Toxic. Vol. 20, pp. 455 to 459, 1982
Printed in Great Britain

0278-6915/82/040455-05$03.00/0
Pergamon Press Ltd

SHORT PAPERS

NITROSAMINE LEVELS IN HUMAN BLOOD, URINE AND GASTRIC ASPIRATE FOLLOWING INGESTION OF FOODS CONTAINING POTENTIAL NITROSAMINE PRECURSORS OR PREFORMED NITROSAMINES

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(*Received 15 December 1981*)

Abstract—In studies of the effect of diet on nitrosamine levels in selected human physiological fluids, volunteers were fed meals containing fish or beef (sources of precursor amines) or bacon (a source of preformed nitrosamines), in combination with spinach and vegetable juice to supply nitrite via possible reduction of nitrate. Blood, urine and gastric contents were sampled periodically for up to 4 hr after feeding. The results of the study indicated that traces of nitrosamines, usually *N*-nitrosodimethylamine, were present in many samples of blood, urine and gastric contents, even after an 8-hr fast. Eating the test meals led to a slight increase in nitrosamine levels in the blood and stomach contents in a few subjects. The data obtained from this study suggest that gastric formation of nitrosamine does not appear to be an important health factor in normal people, since the levels of nitrosamines found in physiological fluids are not markedly increased after eating.

Introduction

Nitrite is present in saliva in fasting subjects, and salivary nitrite levels increase after ingestion of vegetables (Hajimu, Boriboon, Nakamura *et al.* 1975; Tannenbaum, Weisman & Fett, 1976). The nitrite is formed by oral bacterial reduction of nitrate and could serve to nitrosate ingested compounds in the stomach (Spiegelhalder, Eisenbrand & Preussmann, 1976) to produce potentially carcinogenic *N*-nitrosamines. Similarly other sites in the body could also favour nitrosamine formation, particularly under abnormal conditions. Sander & Seif (1969) demonstrated the feasibility of *in vivo* formation by feeding nitrate and diphenylamine to an achlorhydric subject and subsequently detecting *N*-nitrosodiphenylamine in the stomach contents. Volatile nitrosamines have been detected in human blood (Fine, Ross, Rounbehler *et al.* 1977; Lakritz, Simenhoff, Dunn & Fiddler, 1980), in urine (Brooks, Cherry, Thacker & Alley, 1972; Hicks, Gough & Walters, 1978) and in faeces (Wang, Kakizoe, Dion *et al.* 1978).

Several studies to determine the extent of *in vivo* nitrosamine formation have been conducted. Walters, Dyke, Saxby & Walker (1976) passed food slurries through an oral tube and detected up to 0·5 ppb (ng/ml) *N*-nitrosopiperidine in the stomach contents. Fine *et al.* (1977) reported *in vivo* formation of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) in blood from a single subject who had eaten a meal containing bacon, beer, vegetables and bread. The effect of diet on blood-nitrosamine levels was also studied by Yamamoto, Yamada & Tanimura (1980) who fed Japanese diets rich in amines and nitrate to volunteers.

The study reported here was concerned with the effects of diet on levels of nitrosamines (arising either from ingestion of preformed nitrosamines or from endogenous formation) in the blood, urine and gastric contents of volunteers fed several different diets.

Experimental‡

Twenty-one normal healthy male and female volunteers (aged 19–60 yr) participated in this study on one or more occasions. All physiological samples were obtained under the supervision of a gastro-enterologist at a hospital.

Diets. For study A (the preliminary study) and study B, the test meals consisted of 100 ml commercial vegetable juice (containing tomato, carrot, celery, beet, parsley, lettuce, watercress, spinach and ascorbic and citric acids) together with 100 g canned spinach and 125 g fresh cod. The fish fillets were baked at

*Retired.

†Reference to a brand or firm name does not constitute endorsement by the US Department of Agriculture over other products of a similar nature that are not mentioned.

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

Abbreviations: DCM = Dichloromethane; NDEA = *N*-nitrosodiethylamine; NDMA = *N*-nitrosodimethylamine; NMEA = *N*-nitrosomethylethylamine; NPYR = *N*-nitrosopyrrolidine.

300 F for 35 min in aluminium foil without condiments. For study C, 125 g pan-fried beef or 125 g fried bacon was substituted for the fish, rendered fat being removed and not ingested. Samples of all foods were assayed for nitrosamines. The volunteers fasted overnight for at least 8 hr before the study began.

Sampling procedures and protocols. For blood analyses, 10 ml of venous blood was drawn by venepuncture using a syringe and was transferred to and stored in a glass beaker. Commercially available evacuated blood-collection tubes were not used because of the possibility of nitrosamine contamination from the rubber stoppers, as reported by Lakritz & Kimoto (1980). Urine samples were collected in plastic specimen bottles. Samples of gastric contents were obtained by inserting a Levin tube through the nose (or in two cases through the mouth) into the stomach. Neither local anaesthetics nor any liquids were necessary to facilitate the placement of the Levin tube. Samples of 25 ml were aspirated from the stomach for the fasting specimen; the other samples varied in volume. Volunteers were usually in a prone position and were rotated so that sufficient sample could be obtained. All samples collected were immediately made alkaline and frozen.

The preliminary study was conducted to establish appropriate experimental conditions. Due to the paucity of information concerning the rate of formation, transport and metabolism of nitrosamines in humans, it was necessary to determine the optimum times for sample collection. For this study physiological samples were collected from ten subjects before ingestion and at various intervals ranging from 15 min to 4 hr after ingestion of the fish diet. To obtain samples of gastric contents, the Levin tubes were initially placed through the nose or mouth. The food was puréed, since it was assumed that it would be difficult to eat with a Levin tube in place. The presence of an intubation tube in the mouth caused excessive salivation, however, so this practice was discontinued and all samples were collected via nasal intubation. This enabled the subjects to ingest the food intact.

In study B, six subjects (nos 11–16) were all intubated nasally and were fed the diet of fish, spinach and vegetable juice. Samples of gastric aspirate, blood and urine were taken before and 1 hr after feeding and a second blood sample after a further 1 hr. In study C, five volunteers (nos 17–21) were fed the diet in which fried bacon replaced the cod, and in addition to the fasting samples, samples from the stomach were taken 1 hr after the meal and blood and urine samples 1 and 2 hr after feeding. These same subjects participated in a second session in which the sampling schedule was the same but the bacon in the test meal was replaced by a grilled hamburger.

N-Nitrosamine analyses. Samples of blood and gastric contents were analysed for volatile nitrosamines by a modification of a procedure described by Telling, Bryce & Althorpe (1971). *N*-Nitrosomethylethylamine (NMEA; 1·0 ml of a 0·05 μg/ml solution) was added to each sample as an internal standard. Samples were added to flasks containing NaOH, $Ba(OH)_2$ and water and the mixture was distilled. Sodium chloride was added to the distillate, which was then extracted three times with dichloromethane (DCM). The DCM layer was washed with 6 N-HCl and with 5 N-NaOH, dried over anhydrous Na_2SO_4 and concentrated to 1·0 ml in a Kuderna-Danish evaporator flask equipped with a concentrator tube. Urine samples were extracted in a liquid/liquid extractor described by Fazio, White, Dusold & Howard (1973). Sodium chloride and the internal standard (NMEA) were added to the urine sample, which was continuously extracted with DCM for 5 hr. The extracts were concentrated as described above. Food samples (fish, spinach, meats) were digested in alcoholic KOH and then subjected to liquid/liquid extraction with DCM followed by distillation from base.

The DCM extracts were concentrated and analysed with a gas chromatograph interfaced with a Thermal Energy Analyzer (Model 502, Thermo Electron Corp., Waltham, MA), a selective nitrosamine detector. The nitrosamines were separated on a Varian-Aerograph Model 1720 gas chromatograph (Palo Alto, CA) equipped with 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 *N*-nitrosopyrrolidine (NPYR) eluted at 4·2, 5·6 and 14·5 min, respectively. The Thermal Energy Analyzer was operated under conditions similar to those used by Fine & Rounbehler (1975). Samples containing concentrations as low as 0·1 ppb could be detected readily. Presumptive confirmation was obtained by the ultraviolet photolysis procedure described by Doerr & Fiddler (1977). Samples containing sufficient amounts of nitrosamines were also subjected to gas chromatographic–mass spectrophotometric analysis for confirmation. The analyses were performed using a Varian-Aerograph Model 2700 gas chromatograph equipped with a glass column (6 ft × 1/4 in. OD) packed with 15% Carbowax 20 M-TPA on Gas Chrom P connected to a Varian MAT 311A mass spectrometer (Finnigan-MAT, Sunnyvale, CA). The helium flow rate was 15 ml/min; the temperatures of the detector, injector port and gas chromatograph–mass spectrometer interface systems were 200, 200 and 180°C, respectively. Column and operating conditions were similar to those described by Lakritz & Kimoto (1980).

Results and Discussion

Preliminary study

The levels of NDMA found in the analyses for volatile nitrosamines carried out on gastric aspirates from the ten subjects tested in the preliminary studies are presented in Fig. 1. Concentrations of NDMA were considerably higher in the first two subjects, possibly because in these two instances the meals were prepared and puréed several hours before they were consumed and were stored at room temperature, thus providing opportunity for the formation of nitrosamines. In addition to the NDMA detected in the gastric samples from subject no. 2, two of these samples contained 2 and 3 ppb NDEA although this nitrosamine was not detected in the food prior to

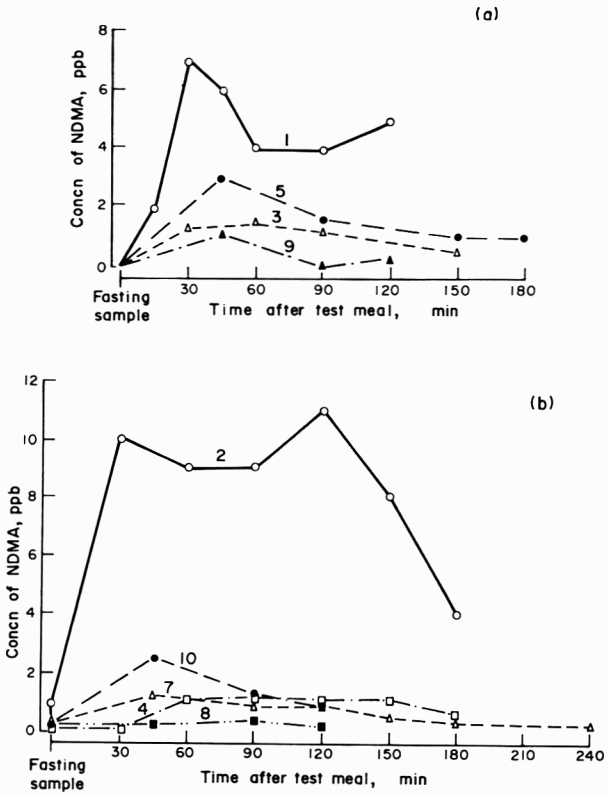

Fig. 1. Concentrations of *N*-nitrosodimethylamine (NDMA) in samples of gastric aspirate taken from test subjects after fasting and at intervals after ingestion of a meal consisting of fish, spinach and vegetable juice. Numbers on graphs (a) and (b) identify the different subjects. No samples could be taken from subject 6.

ingestion. Both NDMA and NDEA were confirmed by mass spectrometry.

Examination of data by linear regression demonstrated that nitrosamine concentrations in the gastric contents reached a maximum between 30 and 60 min after food consumption. Rapid absorption of a nitrosamine (NPYR) in the stomach was previously demonstrated by Mysliwy, Wick, Archer *et al.* (1974) when they introduced $NaNO_2$ and pyrrolidine into the stomach of a fistulated dog. Therefore the subsequent decrease in nitrosamine content in our study was not unexpected.

Blood nitrosamine levels from some subjects participating in the preliminary study are presented in Fig. 2a. In four (subjects 6, 7, 8 and 10) there was an increase in the nitrosamine concentrations; however such increases were not statistically significant when all the data were analysed.

Figure 2b depicts the urinary NDMA levels. Concentrations greater than 1 ppb NDMA were detected only in volunteers 1 and 3. In both of these cases, no nitrosamines were detected in the fasting state, and a progressive increase after treatment suggested possible absorption followed by rapid excretion by the kidney. Urine specimens from the remaining subjects contained less than 0·7 ppb NDMA and were unaffected by the test diet. Statistical evaluation showed no correlation between ingestion and the formation and or excretion of nitrosamines.

Studies B and C

Information obtained in the preliminary study was used as a guide to establish the parameters and techniques to be used in the subsequent investigations. In study B, six subjects (nos 11–16) were all intubated nasally and fed. The sampling schedule used for the six subjects fed fish, spinach and vegetable juice in study B and the results of the analyses of all the physiological fluids and the foods ingested are presented in Table 1.

Ingestion of the sample meal, which presumably contained high concentrations of amines and nitrate, had no apparent influence on *in vitro* nitrosamine formation in the stomach. No statistically significant increases in nitrosamines were detected in the stomach contents or blood. Urinary levels were unaffected, nitrosamines being essentially absent. Only a slight increase of 0·8 ppb NDMA was noted in a single set of blood samples (no. 15). NDMA increased in the gastric aspirate from one subject (no. 16) from a fasting level of 1·4 ppb to 3·7 ppb, but the meal fed in that particular case contained a higher concentration of existing NDMA than any other meal in this series. The decrease in NDEA from 3·8 ppb, in the fasting state, to 0·2 ppb may have been due to dilution in the stomach or to absorption.

To broaden the scope of the study, bacon, a cured food which often contains preformed nitrosamines,

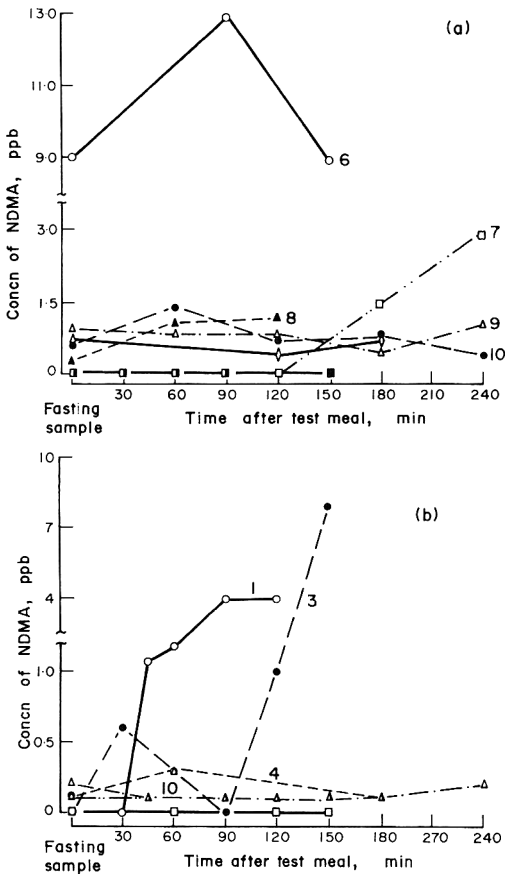

Fig. 2. Concentrations of *N*-nitrosodimethylamine (NDMA) in (a) blood samples and (b) urine samples taken from test subjects after fasting and at intervals after ingestion of a meal consisting of fish, spinach and vegetable juice. Numbers on the graphs identify the different subjects.

was substituted for fish in the sample diet (study C). In an effort to reduce variability, each volunteer participated twice. At the first session volunteers ingested vegetable juice, spinach and fried bacon, which at times contained normally incurred preformed NPYR. A grilled hamburger replaced the bacon at the second session. The results of this study (Table 2) indicated that eating these foods did not significantly alter the levels of nitrosamines in any of the physiological specimens tested. In this study, the gastric contents was sampled 1 hr after the subject completed the meal, and blood and urine were collected at 1- and 2-hr intervals. The concentrations of nitrosamines, if any, in samples from the stomach remained unchanged or increased only slightly after consumption of meals containing nitrosamine or nitrosamine precursors. Whether the increases, when they occurred, were due to ingestion of preformed nitrosamine, or to *in vivo* gastric formation cannot be ascertained. However, the data suggest that the former possibility is more likely to be correct.

Other investigators have conducted studies, but only in blood, to determine the effect of diet on the *in vivo* formation of nitrosamines. Yamamoto *et al.* (1980) observed no effect after feeding eight individuals a Japanese diet rich in nitrate and amines. Kowalski, Miller & Sen (1980) repeated the study by Fine *et al.* (1977), using eight subjects rather than one, and noted on occasion a slight increase in nitrosamines in some volunteers after they had eaten the same test meal, but the difference was less marked than that reported by Fine's group. The data obtained in this study on blood levels of nitrosamines are comparable to these investigations. The results illustrate that there are fluctuations in nitrosamine levels in blood, and point to the need for sufficient sampling, particularly in studies of a biological nature, before definitive conclusions are drawn.

The results compiled in this study indicate that nitrosamine levels in gastric contents, blood and urine from normal people are not significantly affected by ingestion of an ordinary diet containing preformed nitrosamine or potential nitrosamine precursors. The possibility that nitrosamines may be formed and rapidly metabolized or absorbed cannot be completely ruled out, and was not within the scope of this study.

Table 1. *N-Nitrosodimethylamine in physiological samples and in the components of a test meal*

Subject no.	Concn of *N*-nitrosodimethylamine (ppb*)									
	In gastric contents		In blood			In urine		In food†		
	Fasting‡	1 hr§	Fasting‡	1 hr§	2 hr§	Fasting‡	1 hr§	Cod	Spinach	Juice
11	0·4	0·2	0·6	ND	0·4	ND	ND	0·6	ND	0·1
12	0·2	0·3	0·4	0·6	0·4	ND	ND	1·5	ND	0·2
13	0·3	0·2	0·8	0·4	ND	0·2	ND	1·3	0·3	0·2
14	0·1	0·3	—	—	—	ND	ND	ND	0·2	0·1
15	0·3	0·6	0·4	1·2	1·0	ND	ND	0·8	0·3	0·2
16	1·4	3·7	0·6	0·7	0·7	0·2	0·1	3·0	0·6	0·2
	(3·8)‖	(0·2)‖								

ND = None detected

*ng/ml or ng/g, as appropriate.
†As consumed by the subject indicated.
‡Sample taken at the end of an (at least) 8-hr fast, prior to ingestion of the test meal.
§Interval between ingestion of test meal and sampling.
‖Concentration of *N*-nitrosodiethylamine, found only in these samples from subject 16.

Table 2. *Influence of beef or bacon ingestion on the* N-*nitrosodimethylamine content of physiological fluids*

Subject no.	Type of diet	Concn of *N*-nitrosodimethylamine (ppb*)										
		In gastric contents		In blood			In urine			In food†		
		Fasting‡	1 hr§	Fasting‡	1 hr§	2 hr§	Fasting‡	1 hr§	2 hr§	Meat‖	Spinach	Juice
17	Beef	0·2	0·2	ND	0·6	0·4	0·1	0·1	0·3	0·3	0·4	0·3
	Bacon	0·3	0·1	0·6	0·4	0·5	0·1	0·1	0·1	1·0	0·3	0·2
18	Beef	0·1	0·1	ND	ND	ND	0·1	0·1	0·1	0·3	ND	0·2
	Bacon	0·4	0·1	0·3	0·3	0·4	ND	0·1	0·1	1·4	0·5	0·2
19	Beef	ND	0·2	0·2	0·2	0·2	0·2	0·1	0·2	0·9	0·1	0·2
	Bacon	0·2	0·3	0·5	0·6	0·3	0·2	ND	0·3	2·1	0·2	0·2
20	Beef	ND	ND	0·5	0·5	0·4	0·7	ND	ND	0·4	0·2	ND
	Bacon	0·2	0·2	0·5	0·6	0·5	0·4	ND	0·2	1·3	0·2	0·2
21	Beef	0·7	0·2	0·4	0·4	0·4	ND	ND	ND	0·4	0·2	0·2
	Bacon	0·3	0·2	0·5	0·7	0·4	ND	ND	ND	0·9	0·2	0·3

ND = None detected

*ng/ml or ng/g, as appropriate.
†As consumed by the subject indicated.
‡Sample taken at end of an (at least) 8-hr fast, prior to ingestion of the test meal.
§Interval between ingestion of test meal and sampling.
‖The bacon samples in the diets fed to subjects 17, 18 and 21 also contained *N*-nitrosopyrrolidine, at levels of 2·2, 3·5 and 2·2 ppb, respectively.

Acknowledgements—The authors thank C. J. Dooley and E. G. Piotrowski for conducting mass spectral analyses, Franz Goldstein, M.D. for facilitating the *in vivo* portion of the study carried out at Lankenau Hospital, Philadelphia, PA, W. Fiddler for assistance with the manuscript, and the National Cancer Institute for the loan of a Thermal Energy Analyzer under control No. 1-CP55715.

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Fd Chem. Toxic. Vol. 20, pp. 461 to 465, 1982
Printed in Great Britain.

0278-6915/82/040461-05$03.00/0

TARTRAZINE-INDUCED CHROMOSOMAL ABERRATIONS IN MAMMALIAN CELLS

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(*Received 12 January 1982*)

Abstract—Tartrazine (FD & C Yellow No.5) has been shown to induce chromosomal aberrations in fibroblast cells of *Muntiacus muntjac in vitro*. *M. muntjac* cells were exposed to various concentrations of tartrazine (in the 5–20 μg/ml range) and were evaluated for induced chromosomal aberrations after two different periods of culture. Total percentages of chromosomal aberrations were significantly increased above control levels in all experimental groups. The results suggest that further studies are needed to determine the potential mutagenic effects of tartrazine.

Introduction

Tartrazine (FD & C Yellow No.5) is a pyrazoleaniline dye (5-oxo-1-(*p*-sulphophenyl)-4-(sulphophenylazo)-2-pyrazoline-3-carboxylic acid, trisodium salt), which is used to colour candy, soft drinks, drug capsules, hand lotions and many other foods, drugs and cosmetics. Rats fed tartrazine for prolonged periods did not reveal any evidence of toxic reactions (Mannell, Grice, Lu & Allmark, 1958). However, Ershoff (1977) reported that rats fed a 5% level of tartrazine in a purified diet showed marked retardation of growth and a 50% death rate during the 14-day experimental period. Tartrazine has been shown to induce allergic reactions in specific human populations (Neuman, Elian, Nahum *et al.* 1978; Stenius & Lemola, 1976). One third of an allergic population studied was shown to be clinically affected by tartrazine. A correlation has been demonstrated between tartrazine sensitivity and aspirin intolerance (Samter & Beers, 1968; Stenius & Lemola, 1976), up to 44% of aspirin-sensitive patients having been shown to react adversely to tartrazine. The apparent association between tartrazine sensitivity and aspirin intolerance is not yet understood.

In the investigations reported here, a study has been made of the effects of tartrazine on the chromosomes of *Muntiacus muntjac* as reflected in induced percentage increases in chromosomal aberrations.

Experimental

Male *M. muntjac* fibroblast cells (American Type Culture Collection, Rockville, MD) were grown in Eagle's Basal Medium supplemented with 10% foetal calf serum and 100 units kanamycin/ml (GIBCO, Grand Island, NY). The fibroblast cells were treated with 3, 5, 10 or 20 μg tartrazine/ml (Warner-Jenkinson Co., St Louis, MO) for 48 hr in short-term treatment studies. In a second series of investigations, fibroblast cells were exposed to 5 or 10 μg tartrazine/ml for 3 days and were then cultured for an additional 13 days (a total of 16 days in culture).

After treatment with one of the specific concentrations of tartrazine, the experimental fibroblast cells, together with the control cells, were prepared for cytogenetic analysis as previously described (Patterson & Petricciani, 1973). Briefly, the cells in culture were treated with 0·025 or 0·03 μg colcemid/ml for 15 hr. The cells were then harvested, treated with hypotonic medium without serum, fixed in acetic acid–methanol (1:3, v/v) and stained in dilute Giemsa. Aberration counts were made on at least 100 metaphase cells with well-spread chromosomes.

Results

M. muntjac fibroblasts treated with 3 μg tartrazine/ml for 48 hr yielded a 30% incidence of aberrations, compared with 6·03% in the control group (Table 1). In this experimental group, 15% of chromosomes exhibited chromatid breaks (Fig. 1), and there were more chromatid gaps and dicentric chromosomes than in control cells. With increasing concentration of tartrazine for the 48-hr treatment, there was a significant increase in chromosome damage; cells treated with 5 μg tartrazine/ml showed a 46% incidence of aberrant chromosomes, and compared to the controls (Table 1) this group exhibited a high frequency of dicentric chromosomes (Fig. 2).

When the tartrazine concentration was increased to 10 μg/ml for 48 hr, there was an increase (to 53·45%) in the percentage of aberrant chromosomes, as well as an increase in the type of aberrations observed (Table 1). The highest percentage of aberrations occurred as breaks of chromosome No. 3 with and without displacement. Ring chromosomes (Fig. 3) were observed at a low frequency. Cells treated with 20 μg tartrazine/ml for 48 hr exhibited a 33·6% incidence of chromosomal aberrations (Table 1). In this experimental group, 18·8% of the chromosomes were dicentrics; other aberrations included chromatid breaks (7·9%) and chromatid gaps (6·9%).

In *M. muntjac* fibroblast cells treated with 5 μg tartrazine/ml for 72 hr and allowed to remain in culture for an additional 13 days (a total of 16 days), 48% of the chromosomes showed aberrations (Table 2). Chromatid separation was the most frequent type of aberration; breaks and gaps were also observed. Cells

Table 1. *Incidence of chromosomal aberrations in* Muntiacus muntjac *cells exposed for 48 hr to tartrazine in concentrations of 3–20 μg/ml*

Aberration	Tartrazine concn (μg ml)...	Incidence of aberration (%)					
		Experiment 1			Experiment 2		
		0*	3	5	0*	10	20
	No. of metaphases examined...	116	200	103	128	152	101
Chromatid breaks		0	15·0	10·6	0	19·7	7·9
Chromatid gaps		1·72	8·0	9·3	1·5	6·8	6·9
Dicentrics		0	5·0	16·0	0	18·4	18·8
Fusions		0	0	3·3	0	0	0
Rings		0	0	0	0	0·66	0
Polyploids		0	0	0	0	7·89	0
Chromatid separations		4·31	2·0	6·8	2·3	0	0
	Total aberrations (%)...	6·03	30·0	46·0	3·8	53·45	33·6

*Control values.

treated with 10 μg tartrazine/ml for the same culture times (Table 2) exhibited a 25% incidence of aberrations, including endoreduplications, breaks, gaps, dicentrics and chromatid separations.

Discussion

These investigations indicated that *M. muntjac* fibroblast cells exposed to 3, 5, 10 or 20 μg tartrazine/ml showed significant increases in chromosomal aberrations. The experimental cultures exposed to 3 μg/ml exhibited a significant increase in the percentage of aberrations compared to the control group. With a tartrazine exposure of 5 μg/ml for 48 hr, there was a corresponding increase in chromosome damage above that with the 3-μg ml exposure. Similar results were obtained with 10 μg ml, but in the 20-μg ml cultures the mitotic index was considerably lower than those for 3, 5 and 10 μg tartrazine/ml, respectively. Lewis, Patterson & McBay (1981) also noted a decrease in chromosomal-aberration percentages when *M. muntjac* fibroblast cells were exposed to the xanthine dye Rhodamine B (D & C Red No. 19) at a level of 20 μg/ml. Although chromosomal aberration studies with tartrazine have not previously been reported, Yahagi, Degawa, Seino *et al.* (1975) found several azo dyes and their derivatives to be mutagenic carcinogenic and suggested that these effects may involve modification of the DNA.

Cells exposed to 5 μg tartrazine/ml for 3 days and allowed to remain in culture for an additional 13 days did not show a significant decrease in total chromosomal aberrations, but did show a significant decrease in breaks, gaps and dicentrics, compared to the short-term studies. Chromosomal structural changes that are due to lesions (breaks and/or gaps) are discontinuities that can occur as a result of chemical action (Evans, 1962). The vast majority (90–99·7%) of these lesions in a chromosome are rejoined in the original order by repair processes, and the chromosomes reveal no visible structural changes. In the 16-day study with 5 μg tartrazine/ml, a high percentage of chromatid separation accounted for most of the aberrations observed. Cells treated with 10 μg tartrazine/ml for 3 days showed a lower percentage of aberrations than did the 5-μg/ml cultures. Although in this study, 10-μg tartrazine/ml did not increase chromosomal aberrations compared with the 5-μg ml exposure, as was the case in the short-term studies, it did induce

Table 2. *Incidence of chromosomal aberrations in* Muntiacus muntjac *cells exposed for 3 days to tartrazine in a concentration of 5 or 10 μg ml and then cultured for a further 13 days*

Aberration	Tartrazine concn (μg/ml)...	Incidence of aberration (%)		
		0	5	10
	No. of metaphases examined...	137	100	40
Chromatid breaks		0	4·0	10·0
Chromatid gaps		0·7	5·0	2·5
Dicentrics		0	0	7·5
Chromatid separations		2·9	39·0	2·5
Endoreduplications		0	0	2·5
	Total aberrations (%)...	3·6	48·0	25·0

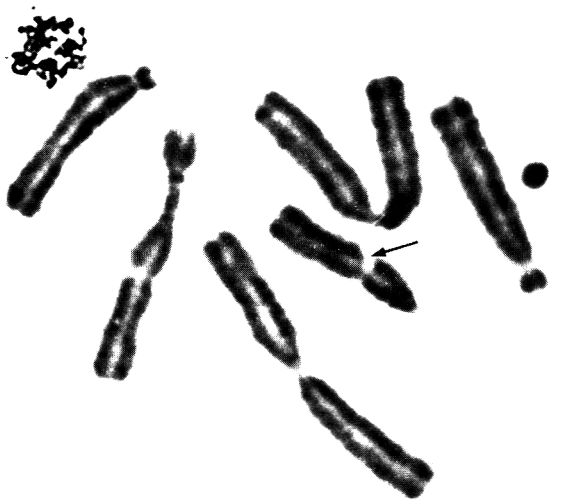

Fig. 1. Chromatid break (arrowed) in chromosomes of a *M. muntjac* cell exposed to 3 μg tartrazine/ml for 48 hr. Giemsa × 1200.

Fig. 2. Dicentric chromosomes of *M. muntjac* cell exposed to 5 μg tartrazine/ml for 48 hr. Giemsa × 1200.

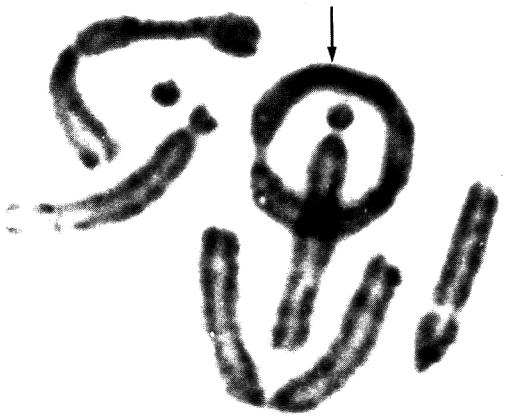

Fig. 3. Ring chromosomes (arrowed) of *M. muntjac* cell exposed to 10 μg tartrazine/ml for 48 hr. Giemsa $\times$ 1200.

endoreduplication, an aberration not observed in the other experimental cultures.

Acknowledgements—This research was supported by NIH Grant RR 08806 and NSF Grant RIM 78-16355.

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Fd Chem. Toxic. Vol 20, pp. 467 to 471, 1982
Printed in Great Britain.

0278-6915/82/040467-05$03.00/0

SHORT-TERM TOXICITY STUDY OF CARNAUBA WAX IN RATS

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(*Received 11 November 1981*)

Abstract—Groups of 15 male and 15 female rats were fed diet containing 0 (control), 1, 5 or 10% carnauba wax or 10% cellulose powder for 13 wk and groups of five rats of each sex were given these treatments, except the 1% carnauba wax, for 2 or 6 wk. Rats given 10% carnauba wax or 10% cellulose consumed more food than the controls but showed no differences in body weight, an effect attributed to the dilution of the diet by non-nutrient test materials. The study showed no treatment-related differences in body weights, water intakes, haematological values, serum-enzyme activities, urinary concentration and 'dilution' tests, organ weights or histological findings. The no-untoward-effect level for carnauba wax in the diet was 10%, which represented a mean intake of approximately 8·8 and 10·2 g/kg body weight/day in males and females, respectively.

Introduction

Carnauba wax is obtained from the leaves of the carnauba palm, *Copernica cerifera*, which grows in north-east Brazil. Purification yields several grades of the wax ranging in colour from dark brown to pale yellow, the latter being the most pure. It is used as a glaze for panned confectionery and in the polishing of enteric coatings on tablets. However most of the imported wax is used in polishes for floors, cars and shoes and in the manufacture of carbon paper.

The pale-yellow grade of carnauba wax has the following categories of chemical constituents: aliphatic esters (average molecular weight 840) 38–40%, *p*-hydroxycinnamic aliphatic diesters (molecular weight 1010–1020) 20–30%, ω-hydroxy aliphatic esters 12–14%, monohydric alcohols 10–12%, *p*-methoxycinnamic aliphatic diesters 5–7%, an uncombined triterpene-type diol 0·4% and unidentified constituents 5–7% (Vancenburg & Wilder, 1970).

Carnauba wax is generally recognized as safe as a miscellaneous or general-purpose food additive under Sec. 182.1978 of the US Code of Federal Regulations and is permitted as a glazing and polishing agent for confectionery in several countries, including Canada, Italy, Norway and West Germany. In the UK its use is restricted to chocolate confectionery, chocolate products and sugar confectionery at a maximum level, in each case, of 200 mg/kg (The Miscellaneous Additives in Food Regulations 1980; Statutory Instrument 1980 No. 1834).

The work reported in this paper was undertaken in the absence of animal data on carnauba wax (Food Additives and Contaminants Committee, 1968). Since it was completed, Babish, Stevens, Cox & Gallo (1978) have reported briefly on two studies in which carnauba wax was fed at dietary levels up to 1% to rats for 1 yr and to dogs for 6 months and no evidence of treatment-related effects was obtained. The rat study included *in utero* exposure and no teratogenic effects or interference with reproductive performance were demonstrated.

Experimental

Test material. Carnauba wax was supplied by Rowntree Mackintosh Ltd, York, and complied with the following *Food Chemicals Codex* (1972) specification: Acid value 2–10; ester value 75–85; melting range 82–86°C; unsaponifiable matter 50–55%; arsenic max 3 ppm; lead max 10 ppm; heavy metals (as Pb) max 40 ppm.

Animals and diet. Rats of a Wistar-derived strain, obtained from a specified-pathogen-free breeding colony (A. Tuck & Son, Rayleigh, Essex), were fed ground Spratts Laboratory Diet no. 1 and given tap-water *ad lib.* The air-conditioned animal room was maintained at 20 ± 2°C.

Experimental design and conduct. Groups of 15 male and 15 female rats, approximately 5 wk of age at the beginning of the study, were housed five to a cage and fed a diet containing 0 (control), 1, 5 or 10% carnauba wax or 10% cellulose powder for 13 wk. The rats fed cellulose powder acted as a control for possible effects due to the replacement of a significant proportion of the diet by a non-nutrient test material. In addition, groups of five rats of each sex and from the same batch of animals were fed 0, 5 or 10% carnauba wax or 10% cellulose powder for 2 or 6 wk. Body weights and water and food consumption were recorded at 2–5-day intervals up to day 14 of the study and weekly thereafter.

At the end of the appropriate feeding period the rats were killed by exsanguination under barbiturate anaesthesia and blood samples were collected for haematological examination and serum analyses. The blood was examined for haemoglobin concentration and packed cell volume. Counts of total erythrocytes and total leucocytes were made. In the absence of

Table 1. *Body weights and food and water consumption of rats fed diet containing carnauba wax at 0–10% or cellulose powder at 10% for up to 13 wk*

Dietary level† (%)	Body weight (g) at wk					Food consumption (g/rat day) at wk					Mean food consumption (g/rat day)	Mean water consumption (ml/rat day)
	0‡	1	5	9	13	0‡	1	5	9	13		
Males												
0	73	125	280	379	433	10·2	15·3	17·7	17·2	18·2	18·1	29·7
1	79	124	283	382	438	10·1	16·4	19·0	18·1	18·1	18·3	24·3
5	73	124	279	374	423	10·8	15·9	19·0	19·1	19·0	18·9	25·0
10	75	127	285	384	437	11·3	18·0	20·4	20·5	21·0	20·5**	27·9
10 CP	74	126	284	378	431	8·9	17·3	21·7	20·1	21·9	21·2**	28·5
Females												
0	68	110	184	222	245	9·2	13·2	14·3	15·5	14·4	14·6	28·0
1	68	110	189	229	251	9·9	14·1	15·6	15·4	16·6	15·0	26·5
5	69	110	181	219	242	10·1	14·1	15·1	15·3	16·2	14·7	24·4
10	68	108	181	214	233	8·7	15·3	16·5	16·8	16·5	16·1**	26·4
10 CP	70	110	180	216	235	10·0	16·0	16·5	16·6	18·0	15·9**	24·5

†Of carnauba wax or cellulose powder (CP).
‡First day of dosing.
Body weight values are the means for groups of 15 animals. Values for food and water consumption are the means for three cages of five animals. There were no significant differences ($P < 0·05$ by Student's t test) between the control and treated rats in respect of body weights. The mean food intakes marked with asterisks differed significantly (**$P < 0·01$; Wilcoxon & Wilcox, 1964) from the control values.

treatment-related changes in erythrocyte counts, the reticulocytes were not examined. Slides for differential counts of leucocytes were prepared for each rat, but the counts were confined to the controls and to animals given 10% carnauba wax or cellulose powder for 2 or 6 wk. The serum was analysed for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

At autopsy any macroscopic abnormalities were noted and the brain, pituitary, thyroid, heart, liver, spleen, kidneys, adrenal glands, gonads, stomach, small intestine and caecum were weighed. Samples of these organs and of lung, lymph nodes, nerve, eye, Harderian gland, salivary gland, trachea, oesophagus, spinal cord, thymus, pancreas, prostate gland, seminal vesicles, colon, rectum, uterus, mammary tissue, urinary bladder and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of the tissues from half the control rats and from those given 10% carnauba wax or 10% cellulose powder were stained with haematoxylin and eosin for histopathological examination.

Urine was collected during wk 2, 6 and 13 of the feeding period and was examined for appearance, microscopic constituents and the semiquantitative estimation of protein, glucose, bile salts, ketones and blood. A urinary concentration test was carried out at the same time. This consisted of measuring the specific gravity and volume of the urine produced in a 6-hr period of water deprivation and in a similar 4-hr period commencing after 16 hr without water. In addition, at wk 6 and 13 the same measurements were made on the urine produced in the first 2 hr after a water load of 25 ml/kg. The number of cells in the urine was counted on this 2-hr sample at wk 13.

Results

There were no deaths in any group and the rats appeared normal throughout the study. The feeding of diets containing carnauba wax at concentrations up to 10% or cellulose powder at 10% had no effect on the body weight in either male or female rats (Table 1) and there were no differences between treated and control rats in the consumption of water (Table 1). Although food consumption in rats given diets containing 10% carnauba wax was higher than that in the controls, similarly high values were noted in the rats given 10% cellulose powder in the diet (Table 1). The calculated mean intakes of carnauba wax were 0·8, 4·2 and 8·8 g/kg body weight/day for the males and 0·9, 4·6 and 10·2 g/kg body weight/day for the females for the 1, 5 and 10% carnauba wax diets, respectively, over the 90-day period.

The only statistically significant difference between control and wax-treated rats in the haematological findings (Table 2) was a higher erythrocyte count at wk 2 in male rats fed diet containing 10% carnauba wax. A similar difference ($P < 0·01$) was seen between the control and the males fed 10% cellulose powder (count $6·15 \times 10^6$/mm^3) again at wk 2 only. The activities of the serum enzymes were similar in test and control rats.

The feeding of carnauba wax or cellulose powder had no consistent effect on the urinary cell excretion or urinary concentration tests (Table 3), the few significant differences being unrelated to dose. For example, at wk 2 in the rats given 10% carnauba wax, the specific gravity of the 0–6-hr urine was lower than the control in the males but higher in the females and the differences were not associated with any differences in volume. At wk 13 the volume of urine produced in the 2 hr following a water load was significantly higher ($P < 0·01$) than the control volume in males fed 1% carnauba wax but not in other male or any female groups. The only other statistically significant difference not shown in Table 3 was an increase ($P < 0·05$) at wk 13 in the mean specific gravity (1·087) of the 16–20 hr urines from females fed 1% carnauba wax. The urines collected from the rats at

Table 2. *Haematological findings in rats fed diet containing 10% carnauba wax for 2, 6 or 13 wk*

Dietary level (%)	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Leucocytes: Total (10^3/mm^3)	Differential (%): N	E	L	M
			Wk 2					
Male								
0	13·8	46	5·20	5·02	17	0	80	3
10	13·5	45	5·88**	3·69	18	0	77	5
Female								
0	13·6	46	6·26	3·48	25	1	70	4
10	13·3	46	6·66	4·18	17	0	82	1
			Wk 6					
Male								
0	14·6	47	7·06	4·11	25	0	73	2
10	15·0	48	7·10	5·49	25	1	71	3
Female								
0	14·9	46	7·13	3·75	22	1	75	2
10	15·6	47	7·46	4·58	31	1	66	2
			Wk 13					
Male								
0	15·0	46	7·24	4·85	—	—	—	—
10	15·3	47	7·47	4·55	—	—	—	—
Female								
0	14·4	45	6·59	2·97	—	—	—	—
10	14·4	45	6·85	2·75	—	—	—	—

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells
N = Neutrophils E = Eosinophils L = Lymphocytes M = Monocytes
Values are means for groups of five rats at wk 2 and 6 and of 15 rats at wk 13.
That marked with asterisks differs significantly (**$P < 0.01$ by Student's t test) from the control value.
Basophils did not constitute more than 0·5% of the white blood cells in any group.

wk 2, 6 or 13 were free from bile, blood, glucose and ketones, while the concentrations of albumin were similar in all groups.

There were a few statistically significant differences between treated and control rats among the organ weights and relative organ weights (Table 4), but only one (a reduction in male adrenal weight at wk 2) was found after the feeding of the highest dietary level of

Table 3. *Results of urinary cell excretion and renal concentration tests in rats fed diet containing 10% carnauba wax for 2, 6 or 13 wk*

Dietary level (%)	Urinary cells (10^3/hr)	Concentration test: Specific gravity 0–6 hr	Specific gravity 16–20 hr	Volume (ml) 0–6 hr	Volume (ml) 16–20 hr	Dilution test (2 hr): Specific gravity	Volume (ml)
				Wk 2			
Male							
0	—	1·038	1·081	2·2	0·4	—	—
10	—	1·023*	1·082	2·0	0·2	—	—
Female							
0	—	1·025	1·085	1·5	0·2	—	—
10	—	1·037*	1·075	1·2	0·3	—	—
				Wk 13			
Male							
0	0·3	1·037	1·074	1·9	0·7	1·004	8·6
10	0·4	1·022*	1·076	2·0	0·7	1·004	7·9
Female							
0	0·1	1·030	1·080	1·5	0·5	1·007	4·4
10	0·5	1·030	1·075	1·1	0·4	1·005	4·8

Results are means for groups of five rats at wk 2 and of 12 rats at wk 13 and those marked with an asterisk differ significantly (*$P < 0.05$ by Student's t test) from the control.
No statistically significant results were obtained in the concentration and dilution tests carried out at wk 6.

Table 4. *Relative organ weights of rats fed diet containing carnauba wax at 10% or cellulose powder at 10% for 2, 6 or 13 wk*

Dietary level† (%)	Terminal body weight (g)	Relative organ weights (g/100 g body weight)												
								Small intestine	Caecum					
		Brain	Heart	Liver	Spleen	Kidneys	Stomach		Full	Empty	Adrenal‡	Gonads§	Pituitary‡	Thyroid‡
Male							**Wk 2**							
0	135	1·28	0·41	3·47	0·41	0·90	0·64	3·92	1·56	0·43	24	1·21	3·7	8·8
10	129	1·30	0·39	3·46	0·42	0·88	0·68	3·40	1·84	0·48	19	1·21	3·8	7·6
10 CP	130	1·30	0·42	3·39	0·37	0·89	0·65	3·95	1·87	0·43	23	1·22	3·4	9·2
Female														
0	110	1·44	0·45	3·58	0·43	0·87	0·79	4·11	1·35	0·48	28	66	5·9	9·6
10	110	1·40	0·50	3·61	0·38	0·91	0·80	4·14	1·67	0·48	29	59	5·8	11·0
10 CP	113	1·44	0·44	3·43*	0·41	0·85	0·82	4·24	2·08*	0·58	25	55*	5·7	9·1
Male							**Wk 6**							
0	292	0·64	0·34	2·90	0·25	0·67	0·45	2·34	1·10	0·31	16	0·98	3·5	5·1
10	284	0·65	0·31	2·78	0·23	0·63	0·46	2·14	1·19	0·31	13	1·01	2·4	4·8
10 CP	264	0·71*	0·31	2·88	0·23	0·69	0·51	2·52	1·40	0·34	15	1·13*	3·0	5·0
Female														
0	182	0·97	0·36	2·68	0·25	0·63	0·57	2·84	1·65	0·38	25	55	5·7	6·9
10	172	1·02	0·38	2·82	0·29	0·70	0·61	3·03	1·58	0·36	27	59	5·8	7·0
10 CP	171	1·01	0·35	2·77	0·27	0·67	0·53	2·85	1·37	0·33	27	46	6·0	7·4
Male							**Wk 13**							
0	426	0·47	0·24	2·30	0·18	0·48	0·35	1·64	0·79	0·22	10	0·75	2·1	3·4
10	426	0·46	0·25	2·35	0·18	0·48	0·36	1·63	0·80	0·22	10	0·75	2·0	3·4
10 CP	415	0·49	0·25	2·38	0·18	0·50*	0·37	1·73	0·86	0·24	10	0·78	2·0	3·3
Female														
0	238	0·78	0·30	2·23	0·22	0·53	0·45	2·04	1·06	0·30	21	29	4·9	5·7
10	227	0·81	0·29	2·54	0·23	0·54	0·46	2·04	1·07	0·30	22	32	5·1	5·5
10 CP	225	0·81	0·30	2·21	0·22	0·54	0·47	2·05	1·18	0·31	22	30	5·0	5·3

†Of carnauba wax or cellulose powder (CP).
‡Relative weights of this organ are expressed in mg/100 g body weight.
§Relative weights of ovaries are expressed in mg/100 g body weight.
Values are means for groups of 15 rats at wk 13 and of five rats at wk 2 and 6 (except for weights of ovaries and male pituitaries at wk 2 and of male thyroids at wk 6, which are all means for groups of four). Values marked with an asterisk differ significantly ($P < 0·05$ by Student's t test) from the control.

carnauba wax. The only organs that showed differences in both actual and relative weights were the ovary, with lower weights in the cellulose-powder group at wk 2 than in the control, and the full caecum, for which the values were higher at wk 2 in females given diet containing 10% cellulose but lower at wk 6 in the females fed 5% carnauba wax and at wk 13 in the males given 1% carnauba wax.

Histopathological examination revealed some inflammatory cell infiltration and a few small areas of focal necrosis in the liver, and some interstitial pneumonitis in the lung, but the incidence and severity of the changes were similar in the control rats and in those given 10% carnauba wax or 10% cellulose powder.

Discussion

The increases in mean food intake by rats given 10% carnauba wax or cellulose were between 9 and 17% and occurred without a parallel increase in body weight. It is probable that these changes in food intake were due to the 10% dilution of the food with a non-nutrient material.

The pattern of changes in the organ weights and relative organ weights did not show any consistent or dose-related effects and thus cannot be attributed to the feeding of carnauba wax. Furthermore, no histological changes attributable to treatment could be detected in any organ.

The isolated increases in the erythrocyte counts at wk 2 did not appear to be related to the treatments, since the differences were evident only in one sex and were not seen in the rats treated for longer periods. The differences in the results of the urinary concentration tests were only marginally significant and were again inconsistently distributed between the sexes and different dose groups. Where a reduction in specific gravity was observed, the corresponding value in the opposite sex was identical to, or greater than, that of the control. Such a pattern does not suggest a treatment-related effect.

It is not considered that these scattered differences can be attributed to the administration of carnauba wax and it is concluded that the no-untoward-effect level in rats given carnauba wax in their diet for 90 days is at least 10%. This dietary level was equivalent to a mean intake of approximately 8·8 and 10·2 g carnauba wax/kg body weight/day in males and females, respectively.

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Fd Chem. Toxic. Vol. 20, pp. 473 to 474, 1982
Printed in Great Britain.

0278-6915/82/040473-02$03.00/0

QUALITATIVE DETECTION OF *N*-NITROSODIETHANOLAMINE IN COSMETIC PRODUCTS

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(*Received 14 January 1982*)

Abstract—A relatively quick and simple procedure is described for the detection of *N*-nitrosodiethanolamine in various cosmetic products. The procedure is based on the irreversible formation of *p*-nitroso-*N*,*N*-dimethylaniline which has a bright yellow colour in basic solution. The compound is formed following extraction of the cosmetic, photolysis of the aqueous extract and the addition of *N*,*N*-dimethylaniline to photolysed extract.

Introduction

N-Nitrosodiethanolamine (NDELA) has been found to cause cancer in laboratory animals (Hilfrich, Schmeltz & Hoffman, 1978) and hence might be carcinogenic in humans. The compounds can be formed by the nitrosation of di- or triethanolamine (Lijinsky, Keefer, Conrad & Van de Bogart, 1972), both of which are ingredients of many cosmetic products. Since NDELA has indeed been detected in some cosmetic products (Fan, Goff, Song *et al.* 1977; Ho, Wisniski & Yates, 1981) it seemed that a simple and inexpensive screening procedure would be useful. A positive result in the screening test would then require a more elaborate quantitative determination.

Experimental

Materials. Cosmetic products were purchased over the counter in the Philadelphia area. Additional samples, known to contain various amounts of NDELA, were obtained from the Division of Cosmetics Technology, Food and Drug Administration (FDA), Washington, DC. NDELA was synthesized according to the method of Preussmann (1962). *N*,*N*-Dimethylaniline was obtained from Aldrich Chemical Co, Milwaukee, WI, and was distilled, in air, prior to use. Reagent-grade activated charcoal was obtained from Durco Corp., New York. All other reagents were ACS reagent grade and were used without further purification. The photolyser was a Rayonet Photochemical Reactor (Southern New England Ultraviolet Co., Hamden, CT).

Procedure. Cosmetic samples were extracted using the procedure of Vohra & Harrington (1981). This is basically a simple extraction using a chloroform-water mixture. The aqueous solution from the chloroform extraction was mixed with about 0·5 g of activated charcoal and allowed to stand for 5 min. The solution was filtered through pre-washed filter paper (Whatman No. 2) to produce a clear, colourless filtrate. A 2·7-ml aliquot was pipetted into a 5-ml culture tube along with 0·3 ml of concentrated HCl. The solution was photolysed for 45 min at 253·7 nm to produce nitrate and nitrite ions (Doerr & Fiddler, 1977). After photolysis 5 μl of *N*,*N*-dimethylaniline was added and the solution was heated in a boiling water bath for 30 min. Upon cooling, NaOH was added to adjust the pH to 9·0 and the colour was noted. The presence of NDELA in the samples was indicated by the yellow colour of the *p*-nitroso-*N*,*N*-dimethylaniline formed. *N*,*N*-Dimethylaniline is known to nitrosate only in the *para* position and to form a yellow compound (Noller, 1965). The colour is enhanced in basic solution since the amine group is then unprotonated. Approximately 0·1 ml of ether was added to the less concentrated samples so that the coloured compound could accumulate in the ether layer and thus be more easily seen.

Results and Discussion

The cosmetic products examined consisted of hand cream, face cream, shampoo, cocoa butter cream, moisturizing lotion, and a make-up remover. All products listed diethanolamine and/or triethanolamine as ingredients. Six of the samples were purchased locally and five were obtained from the FDA, as noted above.

A summary of the results is shown in Table 1. Analyses of all of the samples were run in duplicate and some were repeated four times. One of the six samples purchased locally gave a positive result prior to spiking (sample 1). The remaining five were negative. These were then spiked with NDELA in amounts ranging from 0·78 to 3·28 ppm. All of the spiked samples yielded positive results. Sample 1 was spiked with 885 ppb (b = 10^9) NDELA. This sample then showed the expected increase in colour (sample 1A). One sample (5) spiked with 2·52 ppm NDELA was stored for a day prior to testing. The FDA samples had been stored for several weeks prior to analysis.

Acceptable levels for NDELA in cosmetic products

Abbreviations: FDA = Food and Drug Administration; NDELA = *N*-nitrosodiethanolamine.

Table 1. *Results of analysis of samples of cosmetics for N-nitrosodiethanolamine (NDELA) using a rapid qualitative detection method*

Sample no.*	NDELA concn (ppb)*	Result†
1	?	+
1A	>885	+ +
2	780	+
3	980	+
4	1250	+
5	2520	+ +
6	3280	+ +
a	600	+
b	1018	+
c	5918	+ +
d	7386	+ +
e	1707	+

*Samples 1–6 were purchased locally. Sample 1 gave a positive result before spiking; samples 1A–6 were spiked with the amounts of NDELA indicated. The remaining samples were obtained from the FDA, and the levels of NDELA present were those reported by the FDA. (See text for further details.)

†The results indicate the degree of coloration observed: + = mild, + + = moderate.

have not yet been set by regulatory agencies. The levels used in this study were chosen since they cover the range of permissible levels set for nitrosamines for some products, such as pesticides.

Photolysed NDELA standards analysed by the method of Fan & Tannenbaum (1971) showed that 98–100% of the *N*-nitroso compound was converted to nitrite and nitrate. The ratio of nitrate to nitrite was usually about 7:3. Further reduction of nitrate to nitrite would produce a deeper colour. As seen from Table 1, samples containing as little as 600 ppb yielded a positive result and so no attempts were made to maximize nitrite formation in this study.

The procedure described here is, of course, not specific for NDELA since any nitrosamine would photolyse in the same way. However, the first step in the procedure involves a chloroform extraction. This is used because NDELA is not soluble in chloroform but most nitrosamines and many organic compounds are soluble in chloroform. Thus a false positive could only result from the presence of a substance insoluble in chloroform that could either nitrosate directly or produce a nitrosating species following photolysis. An example of the former type of substance is the nitrite ion but nitrite is rarely used in cosmetics (Rosenberg, Gross, Spears & Rahn, 1980). Whether substances of the latter type are present in cosmetics is not known, but should such a substance be found, the procedure described here would have to be modified.

A finished cosmetic product is often a very complex mixture of many ingredients, and consequently analysis for NDELA is time-consuming and expensive. The procedure described here is simple, does not require a skilled analyst, and could easily be adapted to routine screening of large numbers of samples. Any sample yielding a positive result would than be subjected to the more elaborate analytical procedures.

Acknowledgements—This investigation was supported, in part, by grant No. CA 18618, awarded by the National Cancer Institute, DHEW. The authors would also like to thank Dr Hardy J. Chou of the Food and Drug Administration for his generous supply of analysed samples.

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Fd Chem. Toxic. Vol. 20, pp. 475 to 478, 1982
Printed in Great Britain

0278-6915/82/040475-04$03.00/0
Pergamon Press Ltd

Review Section

REVIEWS OF RECENT PUBLICATIONS

Safety of Foods. 2nd Ed. Edited by H. D. Graham. AVI Publishing Co., Inc., Westport, CT, 1980. pp. xvi + 774. $54.00.

This book was first published as a report of the proceedings of an international symposium on the importance and safety of foods, held in Mayaquez, Puerto Rico in 1967 (*Cited in F.C.T.* 1969, **7**, 635). Changes in food regulations and recent developments in manufacturing processes have prompted the publication of this second edition. In order to up-date the original conference proceedings without convening another conference the structure of the book has been altered from a meeting report to a standard text. The result is a publication almost double the size of the first edition with less emphasis on differences in regulations between countries, and more chapters giving detailed coverage of most aspects of food safety.

The first section is concerned with microbiological problems: food spoilage and contamination by pathogenic micro-organisms. Comprehensive chapters give the background to both topics, and are followed by chapters outlining the particular hazards of bacterial food poisoning, viral contamination and mycotoxins in foods. The section is concluded by a paper on the control of food-borne diseases.

The section entitled "Toxic chemicals in foods" opens with a chapter on food-borne diseases of animal origin which seems a little out of place, and has been very little up-dated. The subsequent contributions are concerned with chemical toxicants, with chapters covering nitrosamines, mercury and other heavy metals, polychlorinated and polybrominated biphenyls, pesticides, antibiotics, and plant and animal toxins. Two contributions are presented on food additives (those intentionally added), describing the types of additive, their function, and correct usage. There is also a completely revised, and very comprehensive, chapter on the safety and wholesomeness of irradiated foods. Finally in this section is a chapter outlining the problems involved in formulating packaging materials for use with foodstuffs.

The concluding section discusses food regulations in the Americas and Canada. It ends with a paper on the safety of food delivery services to schools, which is surely in the wrong section since it deals with microbiological contamination of school meals during food preparation and delivery. There is an extensive reference list for each chapter, but although the contributions have been updated, there are still very many references to work reported 15 years ago or more. Nevertheless, the book will be of value to students of food science and those people seeking a general understanding of the problems of producing safe foods.

Banbury Report 7. Gastrointestinal Cancer: Endogenous Factors. Edited by W. R. Bruce, P. Correa, M. Lipkin, S. R. Tannenbaum & T. D. Wilkins. Cold Spring Harbor Laboratory, New York, 1981. pp. xiii + 458. $78.00

It has long been recognized that gastro-intestinal cancer is a disease with environmental origins, yet difficulties in establishing cause and effect relationships have resulted in the rapid growth of an overlarge literature with many specialist areas. This seventh volume in the Banbury Report series presents the proceedings of a conference held at the Banbury Center and covers many of the factors important in this disease process. The book is divided into sections which allows the reader to locate areas of interest with ease. Transcripts of the participants' discussions presented at the end of many of the papers make useful reading.

The initial sections cover the modifying effects that the gut bacteria and dietary fibre may exert on the disposition of compounds within the gastro-intestinal tract. Together, these sections provide an overview of some of the mechanisms relevant to carcinogen production in the gut lumen. Following this, variations in the host response to these carcinogens are outlined, and a paper is included on alterations in colonic enzymes in premalignant and malignant tissue which might provide a useful diagnostic tool. The effects of chemical inhibitors on neoplastic development are also covered. Further sections are devoted to the isolation, characterization and fate of mutagens occurring in the gut contents, and to the endogenous formation of *N*-nitroso compounds. The effects of bile acids, known promoters of tumour development, are the subject of the penultimate section. Finally, epidemiological results are discussed and guidelines for the use of mutagenesis testing in future studies on human populations are presented.

This book provides a concise coverage of much of the current thinking in the field of gastro-intestinal cancer. The papers are generally of a high standard and clearly written; together they present a very readable overview of many of the relevant factors in this field.

Banbury Report 8. Hormones and Breast Cancer. Edited by M. C. Pike, P. K. Siiteri & C. W. Welsch. Cold Spring Harbor Laboratory, New York, 1981. pp. xi + 491. $78.00.

In many parts of the world breast cancer is the most common malignant neoplasm in women, but despite massive attention from researchers, the aetiology has remained obscure. However, it is known that endocrine factors contribute in an essential way to neoplastic development in the human breast, and the Banbury Conference, held on 26–29 October 1980, brought together epidemiologists, endocrinologists and experimentalists to discuss the science of hormones and breast cancer. The proceedings have now been published in book form as the eighth Banbury

Report, covering 27 presentations grouped into eight sessions. The human problem was the focus of the meeting, and results from animal experimentation were restricted to the last two sessions.

Epidemiological data have shown that three natural factors increase the risk of breast cancer: early menarche, late age at first pregnancy and late menopause. Thus the importance of endogenous hormone exposure is indicated, but the wide variation in the incidence of breast cancer between different countries points to still unidentified environmental factors playing an additional role.

The first four sessions of the conference were concerned with various aspects of the relationship between hormonal profiles and cancer, and oestrogen, progesterone, prolactin and thyroid-hormone levels were discussed in separate presentations. The session on exogenous hormones and cancer covered the possibility of iatrogenic disease. On the whole, reassurance was provided on oral contraceptive use, but high-dose oestrogen replacement therapy has yet to be exonerated. The benefits of modern hormone therapy were also discussed in this session, and the difficulties of comparison with multiple cytotoxic chemotherapy were highlighted. An interesting presentation on the mutagenicity of breast fluids, together with a report on the effect of diet on hormone metabolism comprised the session on other exogenous factors and breast cancer.

Several eminent researchers contributed papers to the session on hormonal aspects of murine mammary neoplasia, reviewing most of the experimental work to date in rats and mice. The critical importance of pituitary prolactin secretion in the genesis and growth of both spontaneous and chemically induced mammary cancer in rats and mice was emphasized and the incompletely understood interaction between prolactin and oestrogens was discussed. In contrast to the rodent studies, a definitive role for prolactin in human breast cancer has not been demonstrated.

The fundamental questions of how hormones exert their effects on cells were left to the final session on mechanisms of hormone action. *In vitro* techniques have been utilized in these investigations, with the surprising result that most hormones associated with growth promotion *in vivo* have little effect on mitogenesis in cultures. Without a better understanding of the physiological action of hormones in growth and development, little progress will be made in elucidating the hormonal role in carcinogenesis.

Although brief discussions following each presentation have been incorporated into the report, the fragmentation and lack of perspective frequently encountered in multi-authored works have not been overcome in this book. Most of the information exchanged at this meeting was already available in the literature, and given this, the failure of the editors to present a synthesis and overview of the subject, or to suggest directions for future work, must be regarded as a serious flaw. However, the book itself maintains the high standards of presentation set by its predecessors.

Principles of Genetic Toxicology. By D. Brusick. Plenum Press, New York, 1980. pp. xix + 279. $25.00.

Some time ago (*Cited in F.C.T.* 1981, **19**, 390) we reviewed a number of books on genetic toxicology. Although some very useful and informative publications were included in the group, it was obvious that none of them constituted a clear and comprehensive introduction to a very difficult and complex field. It was a great pleasure, therefore, to receive for review a book that filled this gap in the available literature.

The subject is introduced with a lucid description of basic genetics and the biological consequences of chemically induced disturbance of the genetic apparatus. These early chapters would perhaps have benefitted from the discussion of a few examples of mutagenic chemicals and the chemistry of their metabolic activation and reaction with cellular components. Presumably in the interests of simplicity, the whole book is remarkably academic in its approach.

The main aims of genetic toxicology are described in chapters on screening chemicals for genotoxic properties, genetic risk evaluation and human and environmental monitoring. The practical aspects of the subject are also well covered with descriptions of laboratory facilities required, safety precautions and a description of most of the test systems likely to be used. A series of sample study designs are included for those who wish to proceed to actual laboratory work.

The book is admirably presented, with a great number of clear and informative tables, diagrams and photographs. The quality of presentation, together with an excellent balance of material, makes this an extremely useful publication for anyone who is interested in gaining an insight into a field of toxicology that has tended to become shrouded in mystery. Teachers and those already working in genetic toxicology will also appreciate the first really comprehensive and concise text on the subject.

Research Monographs in Immunology. Vol. 2. **Mitogenic Lymphocyte Transformation.** By D. A. Hume and M. J. Weidemann. Elsevier North-Holland Biomedical Press, Amsterdam, 1980. pp. xi + 251. Dfl. 122.00.

This book presents the lymphocyte as a model system for studying cell growth and proliferation, particular attention being paid not only to the role of lymphocytes in immunity but to the general applicability of their receptor function to mechanisms of growth control in other cells.

The authors discuss in detail the enormous usefulness of plant-lectin-induced mitogenesis *in vitro* both in the elucidation of immunological function and in permitting detection of changes associated with proliferation generally. Such changes include changes in membrane receptor expression and the authors attempt to correlate increased density of specific receptors to lectins such as concanavalin A during lectin-stimulated mitogenesis with an increased density of insulin receptors in rapidly proliferating fibroblasts. Since lymphocyte proliferation is a process involving the interaction of a number of different types of leucocytes rather than of an individual cell and a mitogen molecule, such comparisons are somewhat forced. However, the authors' postulates are challenging, particularly when they discuss changes in the surface morphology of a cell which would allow it to be recognized as 'foreign' by autologous lymphocytes, possibly as a cell-mediated mitogenic response.

A comprehensive study of the regulation of the glycolytic pathway in lymphocytes during DNA synthesis is given and comparisons are drawn between the regulation of pyruvate dehydrogenase by concanavalin A in lymphocytes and by insulin in adipocytes.

In a chapter on intracellular 'second messengers' the effect of adenosine on cell metabolism is discussed, and it is suggested that an explanation for the immunodeficiency associated with the absence of adenosine deaminase is that adenosine—like prostaglandins, which also elevate cyclic AMP in lymphocytes—acts to induce suppressor T-cells.

Although the authors at times labour to make their points, their concept that fundamental parallels can be drawn between insulin action, lymphocyte mitogenesis and the control of growth in a variety of mammalian cells is a challenging one. Mitogenic activation of lymphocytes has already provided a rapid means of assessment of the ability of lymphocytes to proliferate under a variety of conditions; to be able to use this system to understand better the responses of many diverse types of cells undergoing anabolic activation (such as that induced by the action of trophic hormones) could be an exciting step forward.

Immunologic Defects in Laboratory Animals. Edited by M. E. Gershwin & B. Merchant. Plenum Press, New York. 1981. Vol. 1. pp. xx + 360. $42.50: Vol. 2. pp. xx + 382. $37.50.

Generally this book is devoted to comparisons between animals displaying genetically determined immune diseases and normal animals of the same species. Except for an effort to compare the animal defects with their supposed counterparts in human clinical syndromes, a comparison between species has not been attempted.

The first volume introduces the reader to the concept of immunodeficiency and in particular to those defects associated with the maturation of the immune system. It includes chapters on incomplete immune function in otherwise normal animals and on immunohaematological abnormalities as well as more specifically on the consequences of B cell, T cell and macrophage defects. Genetic regulation of immune responsiveness, inherited dysgammaglobulinaemia and the use of the athymic nude mouse and rat models are among the many topics discussed.

The second volume contains a series of chapters dealing with more complex problems, including subtle changes in effector mechanisms. The chapter on the pathogenesis of autoimmunity in New Zealand mice is particularly laudable in terms of the range of diseases covered, the literature review and the lucid discussion of current theories. The final chapter dealing with cryopreservation techniques is fascinating. It considers the physiological basis of the freezing of mammalian embryos. This at first sight extravagant approach to genetic conservation should allow the development of storage facilities for embryos harbouring one or more genetic defects. The importance of this technique is considerable for both immunological and genetic studies since its utilization will not only provide reference material for comparative studies, but will also protect rare or diverse defects from inadvertent loss.

The contributions to the book are ambitious and authoritative. Although both volumes do concentrate heavily on genetic, anatomical, pathological and functional definitions of the immune defects, they also present theories of the interrelationships between these aspects of immune deficiency and provide practical information about the study of immune defects. Each of the syndromes described has been the subject of systematic study and this book provides a comprehensive picture of contemporary inquiry into a fascinating field of immunology.

Infections in the Immunocompromised Host—Pathogenesis, Prevention and Therapy. Developments in Immunology. Vol. 11. Edited by J. Verhoef, P. K. Peterson & P. G. Quie. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980. pp. viii + 315. Dfl. 80.00.

The meeting from which this book arose was concerned with evaluating the effect of micro-organisms on an individual who has a decrease or absence of one (or more) host defence factors. The symposium was in two parts, one involving the pathogenesis and types of infections seen in immunocompromised patients and the second concerning prevention and therapy.

Infections directly related to antibody deficiency include chronic and recurrent infections of the upper and lower respiratory tract and patients appear to be particularly susceptible to bacteria such as *Streptococcus pneumoniae*. Fungal and viral infections that are sometimes also seen in patients with late-onset immunoglobulin deficiency perhaps reflect associated T cell abnormalities. A particularly interesting chapter suggested that the complement system may play an increasingly important part in host defence processes when immunoglobulins and cellular aspects of the immune system are absent or damaged by cytotoxic therapy. Its critical role, as defined by data obtained in animal models, is to mediate clearance and phagocytosis of the circulating bacteria by macrophages of the liver and the spleen. The virulence of the micro-organism is directly correlated with its requirement for splenic sequestration, and in the absence of normal phagocytic cell function complement cannot provide adequate protection. Discussion on disorders of phagocyte function centred on the neutrophil and included abnormalities due to the cell and abnormalities due to mediators acting on the cell. Patients with disorders of phagocyte function have chronic or recurrent bacterial or fungal infections and this has serious consequences, particularly for children.

Human neonates, particularly those born prematurely, have more immunological defects than occur in any other primary or secondary immunodeficiency and although their state of immunodeficiency is short, it can lead to frequent, severe and occasionally fatal infection. Immune defects associated with malnutrition (both over- and under-nutrition) are dealt with at some length. Animal experiments seem to indicate that a low body weight and enough zinc slows the progressive immunodeficiency that occurs with ageing.

A number of drugs for treating immunodeficiency are discussed as well as the possible use of transfer factor, vitamins C and E and leucocyte transfusions. The final impression is that immunocompromised patients will benefit most from studies on adequate prevention and rational antibiotic therapy.

An Introduction to Radioimmunoassay and Related Techniques. By T. Chard. Elsevier/North-Holland Biomedical Press, Amsterdam, 1978. pp. 241. Dfl. 52.00.

This small book in the series "Laboratory Techniques in Biochemistry and Molecular Biology" covers the basic theory of the radioimmunoassay and related 'binding assays', but concentrates on the practical aspects of the use of this assay in biology.

The large part of the book deals in detail with both the requirements for establishing a sound assay system (preparation of purified and radiolabelled antigen, preparation of antibodies, separation systems etc.) and the means of assessing the characteristics (sensitivity and specificity) of the system. The author should be congratulated on his clear presentation, which includes numerous explanatory diagrams together with many graphs illustrating typical results obtained in the practical situation.

Two chapters are devoted to the organizational aspects of providing a routine assay service and to ways of automating the assay system. These are backed up by appendices that list suppliers of equipment and reagents. Another appendix outlines the safety precautions that should be taken when handling radioactive isotopes.

Some of the "related techniques" promised in the title have been rather glossed over. For instance, the ELISA technique (enzyme-linked immunosorbent assay), which has become increasingly developed for use in biological systems, rates only a brief mention on one page. However, on balance, the author has produced a clear, concise and extremely useful book for those who wish to carry out studies using radioimmunoassay.

BOOKS RECEIVED FOR REVIEW

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Saccharin. A Report by Dr Morris F. Cranmer. Edited by G. H. Scherr. Pathotox Publishers, Inc., 1980. pp. xiii + 586. $38.08.

Gene Function: *E. coli* and its Heritable Elements. By R. E. Glass. Croom Helm Ltd, London, 1982. pp. 487. £19.95 (hardback); £9.95 (paperback).

Banbury Report 9. Quantification of Occupational Cancer. Edited by R. Peto & M. Schneiderman. Cold Spring Harbor Laboratory, New York, 1981. pp. xx + 756. $106.80 ($89.00 in USA).

Mycotoxins and N-Nitroso Compounds: Environmental Risks. Vol. I. Edited by R. C. Shank. CRC Press, Inc., Boca Raton, FL. pp. 285. $74.95 (USA); $84.95 (elsewhere).

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IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 26. **Some Antineoplastic and Immunosuppressive Agents.** International Agency for Research on Cancer, Lyon, 1981. pp. 411. Sw.fr. 62.00.

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Fd Chem. Toxic. Vol. 20, pp. 479 to 483, 1982
Printed in Great Britain

0278-6915/82/040479-05$03.00/0
Pergamon Press Ltd

Information Section

ARTICLES OF GENERAL INTEREST

FAECAL NITROSAMINES

There is considerable evidence that *N*-nitroso compounds may be formed endogenously in man and laboratory animals from nitrite and secondary amines in the presence of bacteria (Fishbein, *Sci. Total Envir.* 1979, **13,** 157; *IARC Monographs* No. 17, 1978). In 1978 it was reported that nitrosamines were present in human faeces (Wang *et al. Nature, Lond.* 1978, **276,** 280; *Cited in F.C.T.* 1979, **17,** 415). It was considered likely that these nitrosamines were produced in the lower gastro-intestinal tract by bacterial action on nitrites (produced by reduction of nitrate by oral or intestinal bacteria) and secondary amines (produced by degradation of amino acids or by bacterial metabolism). Because of the prolonged and continuous exposure that nitrosamines in the faeces must represent, their presence could pose a significant hazard both in the lower gastro-intestinal tract and as a result of absorption. Colon cancer is prevalent in areas such as Western Europe and the USA but much less so in Japan, and seems to be associated with high dietary levels of animal fat and protein. If a relationship between diet and faecal nitrosamines were established it would suggest that nitrosamines might have some role in the aetiology of colon cancer.

Direct measurements of nitrosamines in faeces (and body fluids) are extremely difficult. The concentrations of nitrosamines produced are very small, often ppb (b = 10^9) or less, and serious problems of artefact formation, contamination and data interpretation seem to render much of the published data questionable. Earlier reports of volatile nitrosamines in normal human faeces (Wang *et al. loc. cit.*) have been called into question by some more recent and seemingly more careful studies with negative findings (Archer *et al. Banbury Report 7. Gastrointestinal Cancer: Endogenous Factors.* p. 321. Cold Spring Harbor Laboratory, 1981; Eisenbrand *et al. ibid.* p. 275; Lee *et al. Cancer Res.* 1981, **41,** 3992). However yet another recent study (Suzuki & Mitsuoka, *Nature, Lond.* 1981, **294,** 453) with positive findings demonstrates that this is still very much an open question.

Eisenbrand *et al.* (*loc. cit.*) advocate the use of appropriate controls to validate data on the occurrence of nitrosamines in body fluids, gut contents and faeces. Such controls can be used to prove avoidance of artefact formation or contamination during sampling, storage or analysis. These authors report briefly their analysis of 29 faecal samples from healthy individuals and patients, including some on reduced carbohydrate diets. The samples were analysed immediately after collection to avoid the problems of storage and morpholine was added to indicate any artefact formation. No volatile nitrosamines were found except in one sample from a healthy 18-month-old child and this showed a trace (*c.* 0·5 ppb) of apparent *N*-nitrosodimethylamine (NDMA).

The problems of analysing faeces for volatile nitrosamines were spelt out by Archer *et al.* (*loc. cit.*) as loss or destruction of nitrosamines during storage or analysis, synthesis of nitrosamines from precursors during storage or analysis, contamination of glassware and solvents by nitrosamines and interference by impurities during chromatographic analysis. To overcome some of these problems Archer *et al.* used fresh faeces to avoid storage, spiked samples with *N*-nitrosomethylpropylamine (NMPA) to determine recovery, and added 2,6-dimethylmorpholine to detect artefactual nitrosamine synthesis. They also washed their glassware carefully with dichloromethane to remove all traces of adsorbed *N*-nitrosomorpholine (NMOR) and carried out the analysis using gas chromatography–thermal energy analysis. Twelve faecal samples from healthy adults eating a 'free-choice, western-style diet' were analysed. Recovery of the internal standard NMPA was greater than 75% but no other nitrosamines were detected in any sample. Archer *et al.* repeated the method used by Wang *et al.* (*loc. cit.*) and showed that it was subject to artefact formation. They also investigated the stability of NDMA, *N*-nitrosodipropylamine (NDPA) and NMOR in human faeces by adding 17 μg of each/kg faeces and incubating the mixture at 37°C in anaerobic conditions for up to 4 days. No loss of any of the nitrosamines was detected.

Further work by the same group is described by Lee *et al.* (*loc. cit.*) who carried out anaerobic incubations of human faeces with nitrosamine precursors. Samples of faeces (from individuals eating western-style diets) were incubated for 24 or 48 hr at 37°C with a secondary amine (200 mg dimethylamine, dipropylamine or morpholine/kg) or nitrite (up to 500 mg/kg) or a combination of the two. Nitrosamines were only detected when both a secondary amine and nitrite were included and the rate of nitrosation depended on the basicity of the amine. When fresh faeces were used the levels of nitrosamines formed were only 2–20% of those formed when the faeces were autoclaved. When incubations were carried out with nitrate (up to 1000 mg/kg faeces) and morpholine (up to 2000 mg/kg), NMOR was not produced in autoclaved faeces but was produced in small amounts in fresh faeces. These results seem to suggest that faecal micro-organisms inhibit nitrosamine formation rather than catalyse it. Klubes *et al.* (*Fd Cosmet. Toxicol.* 1972, **10,** 757) reached the opposite conclusion but the conditions of their experiment were quite different and Lee *et al.* (*loc. cit.*) considered that

Klubes *et al.* may have observed chemical nitrosation accelerated by the decrease in pH produced by microbial degradation of glucose (which was included in the incubation mixture). Thus Lee *et al.* took the view that although bacteria isolated from human faeces are capable of forming nitrosamines, faeces do not provide an appropriate ecosystem for them to do so. Their data indicate that nitrosamines are not formed at concentrations of less than about 200 mg nitrite or 500 mg nitrate/kg faeces even at high concentrations of amines, and recent reports (e.g. Archer *et al. loc. cit.*) indicate that the levels of nitrite and nitrate in normal human faeces are generally less than 1 mg/kg. [One possible flaw in this argument is that the faeces used for the nitrate/nitrite determinations came from individuals on a 'free-choice, western-style diet' and it is possible that the individuals involved might have a relatively low nitrate intake.] Nevertheless Lee *et al.* do consider it possible that small amounts of nitrosamines might be synthesized close to the intestinal wall where the microenvironment might be aerobic.

If endogenous nitrosation resulting in faecal nitrosamines does take place then it seems very likely that the levels of various dietary components would have a considerable influence on faecal nitrosamine levels. Suzuki & Mitsuoka (*loc. cit.*) have found detectable levels of five nitrosamines in faeces and have demonstrated the effects of different diets on these levels. Their subjects were healthy Japanese males. Three subjects were given a typical Japanese diet for 4 days, then a mixed Japanese diet (made up of items from western, Chinese and Japanese types of diets) for 3 days, a 'balanced western-style' diet for 8 days and then the typical Japanese diet for a further 6 days. In a separate experiment four subjects were given a long-term mixed Japanese diet and then a high-fat, high-meat diet for 4 days. The main component of the typical Japanese diet was boiled rice with some fish and vegetables providing *c.* 2 kcal, 15% protein (mainly cereal protein), 20% fat, 65% carbohydrate, 75 mg vitamin C and 450 mg nitrate. The staple food of the 'balanced western-style' diet was meat, mainly beef, with some bread and vegetables providing *c.* 2·8 kcal, 25% protein, 55% fat, 20% carbohydrate, 70 mg vitamin C and 360 mg nitrate. The mixed Japanese diet provided 2·4 kcal, 20% protein, 30% fat, 50% carbohydrate, 90 mg vitamin C and 250 mg nitrate, while the high-fat, high-meat diet provided 3·0 kcal, 30% protein, 65% fat, 5% carbohydrate, 15 mg vitamin C and 50 mg nitrate.

NDMA, *N*-nitrosodiethylamine (NDEA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR) were detected in the faeces of the subjects. The faecal samples were analysed immediately after collection. Control experiments demonstrated that the reagents and glassware used were not sources of contamination and that nitrosamine yield was not affected by the addition to the samples of 1 M-sulphamic acid (which decomposes nitrite), 50 mM-ascorbic acid (which inhibits nitrosation) or 0·1 M-sodium nitrite. Thus the authors claim that their experiments indicated that there was no artificial formation of nitrosamines during analysis or storage. One fundamental difference between this method and that used by Archer *et al.* (*loc. cit.*) was that these authors attempted to improve the distribution ratio for dichloromethane extraction by salting out and alkylation of the faecal samples. This may help to extract nitrosamines that may be contained in undigested food or bound chemically or physically to some compounds.

Each of the subjects had a similar pattern of nitrosamine levels. On the typical Japanese diet faeces contained NDMA, NDBA and NPYR at levels below 1·7 μg/kg. After 3 days on a mixed Japanese diet NPIP was also present and NPYR increased. The balanced western-style diet produced marked increases in nitrosamines which reached a maximum total mean level of 26·6 μg/kg faeces, and NPIP was particularly increased, with a maximum mean level of 13·3 μg/kg. NDMA, NPIP and NPYR were always present in the faeces but after a few days on the western-style diet NDBA was no longer found. The levels of nitrosamines in the faeces decreased gradually when the individuals returned to a typical Japanese diet. However in the group given the long-term mixed Japanese diet faecal nitrosamine levels decreased when they were transferred to the high-fat, high-meat diet. The authors consider that the balanced western-style diet provides sufficient quantities of both dietary nitrate and amino acids or lipids for the production of substantial levels of faecal nitrosamines. Another possibility not mentioned by the authors might be that the changes in the faecal flora produced by the different diets might account in part for the differences in nitrosamine levels.

The doubts surrounding the available methods for the accurate determination of faecal nitrosamines make it impossible to judge the significance of the results of Suzuki & Mitsuoka (*loc. cit.*) or indeed of the contradictory negative findings. However, even if Suzuki & Mitsuoka have only demonstrated artefactual nitrosamine formation they have found a clear diet-related effect which might represent increased faecal levels of certain nitrosamine precursors. Both the question of the presence of nitrosamines in faeces and (if they are present) of the implications for man have yet to be answered.

NTA—EPIGENETIC TUMORIGENICITY?

It was recently reported that nitrilotriacetic acid (NTA) was to be re-introduced into detergent formulations in small areas of the USA near the Great Lakes (*Pesticide and Toxic Chemical News* 1981, **9** (22), 16). This followed the EPA's sanction of the use of the compound in laundry detergents (*ibid* 1980, **8** (27), 8) and its rejection of a citizen's petition for a ban on the manufacture and distribution of NTA

(*Federal Register* 1980, **45,** 72778). However these moves follow a period during which NTA has had a rough passage through American regulatory channels.

NTA—a chequered history

In the 1960s concern arose about the effects of phosphates derived from household and other detergents on the ecological balance of natural waters. Phosphates were causing eutrophication: stimulating the growth of algae and weeds and thus promoting the deoxygenation of waters and the death of other life forms in lakes and rivers. NTA was developed to replace phosphates as a builder and metal-sequestering agent and showed considerable promise. NTA is biodegradable under aerobic conditions, although there is conflicting evidence about whether biodegradation occurs at low temperatures (*c.* 5°C; *Cited in F.C.T.* 1971, **9,** 888; Eden *et al. Water Res.* 1972, **6,** 877; Thayer & Kensler, *CRC Crit. Rev. envir. Control* 1973, **3,** 375). The amounts of nitrogen that will result from its biodegradation and enter water systems are not thought likely to cause significant eutrophication (Federal Register 1980, **45,** 72779; Thayer & Kensler, *loc. cit.*).

The use of NTA in detergents in the USA ran into difficulties in the early 1970s. It was reported that NTA given in conjunction with cadmium or methylmercury caused more severe embryotoxic effects and a higher incidence of congenital malformations than did the metals administered alone (*Cited in F.C.T.* 1971, **9,** 889). The detergent manufacturers agreed to phase out the use of the new chelating agent (*Chemical and Engineering News* 1971, 4 January, p. 15). Later studies provided no evidence of adverse effects from *in utero* exposure to NTA (*Cited in F.C.T.* 1973, **11,** 675). Fears about the accumulation of NTA in bone and about the possibility that it could be converted to *N*-nitroso compounds (*ibid* 1971, **9,** 887) also seemed to be unfounded (*ibid* 1973, **11,** 675; *ibid* 1974, **12,** 421). In the UK, the Standing Technical Committee on Synthetic Detergents concluded in its 12th report (Department of the Environment, HMSO, London, 1971) that the problem of the possible carryover of heavy metals from sewage to drinking-water supplies by undegraded NTA had not been resolved, and recommended against the use of NTA in detergents. The Committee's view was unchanged in its final report in 1980 (HMSO, London, 1980). However in Canada, where the biodegradation of NTA may be limited by low temperatures, NTA has been used in detergents since 1970 with little evidence of adverse environmental effects (*Ecochem Bulletin* 1979, no. 143).

NTA had been found to cause nephrosis in rats at high dose levels (*Cited in F.C.T.* 1971, **9,** 888; *ibid* 1973, **11,** 495), but in a later NCI study (*Federal Register* 1977, **42,** 25534) high doses of NTA were also shown to induce tumours of the urinary tract. In sanctioning the re-introduction of the use of NTA in laundry detergents (*Pesticide and Toxic Chemical News* 1980, **8** (27), 8) the EPA concluded that the use levels and consequent risks from exposure were low and that at the levels likely to be present in drinking-water the highest probability of tumour formation in rodents was estimated as about one in two million. Since the results of the NCI bioassay became known, a considerable amount of work has been published by researchers at Procter and Gamble's Miami Valley Laboratories on the effects of NTA on the urinary tract of rats and mechanisms for the carcinogenicity of NTA at high dose levels have been proposed.

Tumorigenicity of NTA

In the NCI bioassay (*Federal Register* 1977, **42,** 25534) there was a significant incidence of tumours of the kidney and urinary tract (including transitional cell tumours of the urinary bladder) in male and female Fischer F344 rats given diets containing 2% of the trisodium salt of NTA ($Na_3NTA.H_2O$) for 2 yr or 1·5% of the free acid for 18 months. B6C3F1 male mice given the free acid at the same level for 18 months also showed a significant increase in the incidence of kidney tumours. No statistically significant increase in the incidence of renal or urinary tract tumours was observed in rats fed lower doses of $Na_3NTA.H_2O$ (0·2 or 0·02% for 2 yr, or 1·5 or 0·75% for 18 months) or NTA as the free acid (0·75% for 18 months) or in mice given 0·5 or 0·25% $Na_3NTA.H_2O$ or 0·75% NTA for 18 months. Goyer *et al.* (*J. natn. Cancer Inst.* 1981, **66,** 869) gave male Sprague–Dawley albino rats 0·1% $Na_3NTA.H_2O$ in their drinking-water for 2 yr. [Drinking-water consumption was not determined. Anderson *et al.* (*Fd Chem. Toxic.* 1982, **20,** 105) gave young adult rats (200 g) the same dose of $Na_3NTA.H_2O$ in the drinking-water for 3 wk, and measured food and water consumption. They calculated this dose to be equivalent to 0·2% in the diet (*c.* 2 ml water consumed/g feed consumed).] No bladder tumours were observed, but 25 out of 183 rats developed renal adenomas and four developed renal adenocarcinomas. Five out of 186 control rats had renal adenomas and none developed adenocarcinoma. In earlier studies no increase in the incidence of tumours of the urinary tract was reported in Charles River CD rats given $Na_3NTA.H_2O$ at 0·03, 0·15 or 0·5% in the diet for 2 yr (*Cited in F.C.T.* 1973, **11,** 521; Nixon *et al. Toxic. appl. Pharmac.* 1972, **21,** 244), or in MRC rats given 0·5% NTA in their drinking-water for 5 days/wk for 84 wk (*Cited in F.C.T.* 1974, **12,** 423), although in the latter study there was a high incidence of tumours of various types in all groups including the controls.

An epigenetic mechanism of tumorigenicity?

Kramers (*Mutation Res.* 1976, **40,** 277; *Cited in F.C.T.* 1977, **15,** 496) found that NTA was not mutagenic in *Drosophila melanogaster* and cited other mutagenicity studies in which negative results were obtained with NTA. In rats (*ibid* 1971, **9,** 265) and dogs (*ibid* 1973, **11,** 675; Budny & Arnold, *Toxic. appl. Pharmac.* 1973, **25,** 48) more than 70% of an ingested dose of NTA is absorbed, but in humans only about 12% is absorbed. The monkey and to a lesser extent the rabbit show similarly low absorption of NTA (Michael & Wakim, *ibid* 1971, **10,** 407). In all of the species examined so far, no metabolism of absorbed NTA occurs (although cation exchange does take place) and excretion is via the urine (*Cited in F.C.T.* 1971, **9,** 888; *ibid* 1973, **11,** 675; *ibid* 1974, **12,** 421). Renal clearance of NTA is by filtration with no evidence of either tubular cell secretion or resorption (Anderson *et al. loc. cit.*). If NTA is not genotoxic and

is not metabolized to a genotoxic form how does it exert its tumorigenic effect? Anderson *et al.* (*loc. cit.*) have proposed that it is by an epi(non)genetic mechanism resulting from tissue damage induced by the imbalances in cation disposition caused by high doses of NTA.

Kidney tumours. High dietary levels of $Na_3NTA.H_2O$ cause nephritis, nephrosis and increased urinary excretion of Zn (*Cited in F.C.T.* 1973, **11,** 521; *Federal Register* 1977, **42,** 25534). Tumorigenesis has been observed only at doses of NTA that cause morphological changes in the proximal convoluted tubules (PCTs). It has been suggested (Anderson, *Fd Cosmet. Toxicol.* 1981, **19,** 639; Anderson *et al. loc. cit.*) that the changes in PCT morphology are the result of altered Zn metabolism induced by NTA. Anderson (*loc. cit.*) showed that when a sufficient dose of NTA is given to increase the concentration of NTA in the plasma ultrafiltrate (UF_{NTA}) to greater than about 20 μM, then an increase occurs in the concentration of Zn in the ultrafiltrate (UF_{Zn}), probably by the formation of a Zn–NTA complex. The PCTs resorb the majority of the Zn, but not the NTA, leading to increased renal tissue Zn concentration. If the amount of Zn in the diet is restricted then the extent of PCT toxicity is reduced. Anderson *et al.* (*loc. cit.*) propose that Zn affects two sequences of morphological changes in the PCTs. The initial response to the increased Zn is a vacuolization of the PCTs, which, if dosing continues, can lead to a sequence of simple vacuolated cell hyperplasia, hyperplastic nodules and adenomatous hyperplasia. Zn also exacerbates age-related nephrosis, which commonly occurs in rats, progressing to non-vacuolated cell hyperplasia and adenomatous hyperplasia. Each of these steps is dependent upon the availability of Zn in the plasma. If NTA dosing is continued at a level sufficient to alter Zn disposition then both sequences of morphological changes can finally result in PCT neoplasia. Anderson and his co-workers argue that doses of NTA that are insufficient to raise UF_{NTA} to >20 μM and to increase urinary Zn levels ($\leqslant 0{\cdot}03\%$ $Na_3NTA.H_2O$ in the diet in chronic studies, $\leqslant 0{\cdot}073$ mmol/kg body weight by gavage in a single dose) will not alter Zn metabolism and will not therefore induce tumours in rat kidneys.

Transitional cell tumours of the bladder. In the NCI bioassay rats given high doses of $Na_3NTA.H_2O$ or H_3NTA developed transitional cell neoplasms of the urinary bladder. High doses of NTA also resulted in dose-dependent increases in urinary Ca, crystalline NaCaNTA in the urine and haematuria (Anderson & Kanerva, *Fd Cosmet. Toxicol.* 1979, **17,** 137). They also led to erosive alterations and hyperplasia of the bladder wall observable under the light or electron microscope (Anderson *et al. loc. cit.*). A comparison of the effects of NTA dose (given in the diet) on the amount of insoluble NTA in the urine (determined in subchronic feeding studies) and the incidence of bladder neoplasms has shown a similarity, suggesting a relationship between the two phenomena (Anderson, *Fd Cosmet. Toxicol.* 1980, **18,** 59). It seems that high doses of NTA (>40 μmol/g diet or $>0{\cdot}76\%$ H_3NTA in the diet) result in urinary concentrations of NTA greater than those of divalent cations (M^{2+}) and that Ca is extracted from the bladder wall to combine with the uncomplexed NTA (Anderson *et al. loc. cit.*). It has been shown that feeding a dose of $1{\cdot}5\%$ H_3NTA (a dose that induced bladder neoplasms in the NCI study) is associated with a 50% decrease in the bladder tissue concentration of Ca, but with little change in Mg, Zn, Na or K. When an NTA (as Na_2NTA) concentration (400 μmol/day) which would provide a urine with $[NTA] > [M^{2+}]$ was infused directly into the bladder of rats the levels of Ca in the bladder tissue decreased and urinary Ca increased. At a lower dose of NTA (200 μmol/day, to provide urine $[NTA]:[M^{2+}]$ *c.* 1) there was no decreased bladder tissue Ca although urinary Ca was increased. Urinary Zn was increased to a similar extent by both infusion levels of NTA. Direct infusion of a high dose of Na_2NTA (400 μmol/day) resulted in a severe neutrophil response, epithelial ulceration and erosion and sites of hyperplasia. When an equivalent dose of $CaNH_4NTA$ was infused there was only a mild to moderate neutrophil response and some epithelial thickening (Anderson *et al. loc. cit.*). These studies indicated that bladder tissue and its blood supplies can provide some of the increased urinary Ca and Zn observed in NTA feeding studies. At very high NTA doses, which lead to the presence of uncomplexed NTA in the urine, Ca (but not Zn) is extracted from bladder epithelium faster than it can diffuse into the bladder tissue from the circulating blood.

Bladder tissue cultured in a medium low in Ca shows hyperplasia and endophytic growth of the epithelium. In the absence of Ca there is extensive epithelial cell erosion, probably through desmosome disruption (Reese & Friedman, *Cancer Res.* 1978. **38,** 586). Anderson and his colleagues suggest that similar changes may occur in the bladder epithelium of rats in which Ca depletion of the bladder wall has been induced by high doses of NTA. The prolonged toxic insult may finally lead to neoplasia.

Conclusion

It seems that NTA is tumorigenic at high dose levels, but not at lower dose levels. On the basis of the amount of NTA present in drinking-water (2·82 μg/litre) and a consumption of 35 ml of water/kg/day, Anderson *et al.* (*loc. cit.*) have calculated that humans in Canada will be exposed to 0·1 μg NTA/kg/day. They contrast this with the dose of NTA at and below which UF_{Zn} concentrations are not increased and which they suggest will not therefore cause PCT toxicity—1 μmol/g diet (= 50 μmol/kg body weight/day or 9550 μg/kg/day). The urinary NTA excretion that must be attained for uncomplexed NTA to be present in human urine is >140 μmol/kg body weight/day. The urinary NTA excretion that will result from ingestion of NTA at levels equivalent to those in Canadian drinking-water is $0{\cdot}6 \times 10^{-3}$ μmol/kg/day. Thus it appears, at least on the basis of the work of Anderson and his co-workers, that the levels of NTA to which humans are likely to be exposed through its use in detergents will not have any adverse effects.

The epigenetic mechanisms of tumorigenesis that have been proposed are interesting, but not yet proven. In some cases the evidence is based on studies using only a very limited range of doses and/or very small numbers of animals, amongst which there have sometimes been wide variations in the parameters

studied. In the long-term studies in which tumorigenesis was observed urinary Zn excretion was not measured. Although in the NCI study transitional cell tumours of the urinary bladder occurred, no such tumours were found in the study by Goyer *et al.* (*loc. cit.*). However this may be explained in part by the use of a different strain of rat. In the Goyer *et al.* study the pathology of kidneys is comprehensively described. The incidence and severity of nephritis at the end of the study were similar in the rats given drinking-water containing 0·1% NTA and the controls, although the NTA-exposed animals showed an increase in hyperplasia. This could be construed as evidence that high doses of NTA exacerbate age-related nephrosis. In the NCI study hydronephrosis and nephritis were observed at high doses, and nephritis was found at lower doses. Noting that detailed studies of kidney pathology were not recorded specifically in the NCI report, Alden & Kanerva (*Fd Chem. Toxic.* 1982, **20**, 441) have reviewed the slides of the renal cortex of the treated male rats in the NCI study. They report that the results of this review support the hypothesis of two pathogenic pathways leading to tumour formation in rats treated with high doses of NTA.

[M. E. Hodges—BIBRA]

Fd Chem. Toxic. Vol 20, pp 485 to 492, 1982
Printed in Great Britain

0278-6915/82/040485-08$03.00/0
Pergamon Press Ltd

ABSTRACTS AND COMMENTS

Is EDTA teratogenic?

Schardein, J. L., Sakowski, R., Petrere, J. & Humphrey, R. R. (1981). Teratogenesis studies with EDTA and its salts in rats. *Toxic. appl. Pharmac.* **61,** 423.

Teratogenicity studies with the chelating agent ethylenediaminetetraacetic acid (EDTA) and its salts have produced variable results. When given by intramuscular injection to rats on day 6 or 10 of gestation, 20 or 40 mg/kg caused maternal toxicity and foetal deaths, and polydactyly and tail defects were observed in 9% of the offspring (Tuchmann-Duplessis & Mercier-Parot, *C.r. hebd. Séanc. Acad. Sci. Paris*, Ser. D 1956, **243,** 1064). No evidence of teratogenicity was found in rabbits given 0·1 or 3% EDTA by ocular administration on days 6–18 of gestation, but the higher level was severely embryotoxic (Gasset & Akaboshi, *Investve Ophthal.* 1977, **16,** 652). Dietary levels of 2 or 3% disodium EDTA, given to rats throughout pregnancy or on days 6–21 or 6–14 of gestation, led to maternal diarrhoea and foetal malformations; however, these could be prevented by dietary supplementation with zinc, suggesting the EDTA chelation of this essential metal may have been responsible for its teratogenicity (*Cited in F.C.T.* 1972, **10,** 700). That lower levels may be ingested with impunity was shown by earlier rat feeding tests, in which intakes of the calcium disodium salt up to 250 mg/kg body weight/day were without effect over four generations (*ibid* 1963, **1,** 281) and two generations tolerated dietary levels of 0·5 or 1·0% of disodium EDTA, although rats fed diet containing 5% failed to produce litters (*ibid* 1964, **2,** 763).

In a comparative study of administration of disodium EDTA by different routes on days 7–14 of gestation, rats fed 3% in the diet (average intake 954 mg/kg/day) showed severe maternal toxicity (but no maternal deaths) and a 71% incidence of malformations, whereas 625 or 750 mg/kg given by gavage twice daily (1250 or 1500 mg/kg/day) was lethal to a high proportion of the dams but produced a lower incidence of malformations (20·5% at 1250 mg/kg/day). By subcutaneous injection, 375 mg/kg/day was lethal to 24% of the dams but did not produce a significant number of malformations. These differences in relative toxicity and teratogenicity were attributed to variations in absorption into the circulation, interactions with essential metals and/or stress associated with administration (Kimmel, *Toxic. appl. Pharmac.* 1977, **40,** 299).

The effects of oral administration of EDTA and its disodium, trisodium, calcium disodium and tetrasodium salts have been further explored by Schardein *et al.* (cited above). Rats were given these compounds in equimolar doses equivalent to 1000 mg EDTA/kg/day by gastric intubation on days 7–14 of gestation, either in solution or suspension in 0·2 M-phosphate buffer. Diarrhoea was evident in all groups, its incidence ranging from 10% in the calcium disodium EDTA group to 90% in the tetrasodium EDTA group. Transient depression of activity occurred in a few animals and three dams died during treatment with disodium EDTA. Food intake was slightly decreased during treatment in all groups, and weight gain was depressed, particularly in the EDTA and disodium and tetrasodium EDTA groups, but recovery occurred after treatment was concluded. When the animals were killed, on day 21, no differences between treated and control animals were evident in litter size, sex ratio of offspring, foetal weights, post-implantation loss or the incidence of gross or skeletal malformations. Of 1084 pups in the treated groups 24 were abnormal, compared with 0/237 from dams given the vehicle only and 8/278 untreated controls.

The contrast between these negative findings and the teratogenic effects reported earlier from daily doses of 1250 mg disodium EDTA/kg also given by gavage (Kimmel, *loc. cit.*) was puzzling, but may have been related to the slightly higher dose used in the latter case. Alternatively it is speculated that the affected animals may have suffered from a zinc deficiency caused by Kimmel's use of deionized water, a semi-purified diet and nonmetallic caging. The dose of 1000 mg/kg administered daily in the present study was far above the maximum intakes provided by US diets, calculated as about 0·4 mg/kg/day.

Keep eating the carrots

Shekelle, R. B., Lepper, M., Liu, S., Maliza, C., Raynor, W. J., Rossof, A. H., Paul, O., Shryock, A. M. & Stamler, J. (1981). Dietary vitamin A and risk of cancer in the Western Electric Study. *Lancet* **II,** 1186.

Recently we reviewed some of the recent evidence that high vitamin A and/or β-carotene intake is linked with decreased incidence of cancer, especially lung cancer (*Cited in F.C.T.* 1982, **20,** 333). A further study adding to the evidence that carotene intakes are negatively correlated with lung-cancer risk has now been published.

The study group comprised 2107 men aged 40–55 yr who had all worked for the Western Electric Company in Chicago for at least 2 yr in occupations associated with the manufacture of telephones and related products. Detailed information about food consumption during the preceding 28 days was obtained by interview and by questionnaire initially between October 1957 and December 1958 and then again 1 yr later. During the initial study the data were analysed to determine the usual daily intake of individual nutrients including vitamin A. Retinol and carotene intakes had not been analysed separately and the complete data needed for reanalysis of the two vitamin A sources were no longer available. However, subsequently estimates indicating the consumption of 26 separate foods or food groups were available and the estimates for the major retinol and the major carotene sources were used to provide an

estimate of the relative contributions of retinol and carotene to the total vitamin A intake. The men were re-examined annually until 1969 and 9 yr later death certificates were obtained for those that had died, and questionnaires were answered by almost all of the 1546 survivors. Medical records were obtained for 243 of the 285 men with malignant neoplasms. Sufficient information was available to include 1954 men in the final analysis.

The mean estimated index of carotene intake was 5543 ± 2769 (1 SD) IU day and that for retinol was 4734 ± 3196 (1 SD) IU day. The estimated carotene index was inversely related to lung-cancer incidence but not to the incidence of all other carcinomas grouped together. The estimated retinol index was not significantly associated with the incidence of lung or other cancers. The carotene index did show a small negative correlation with the numbers of cigarettes smoked/day and with the duration of smoking although it did not correlate with age or serum cholesterol. None of these factors correlated with the retinol index. Both dietary carotene level and duration of smoking were associated with the incidence of lung cancer. However, the carotene index had a significant inverse association with lung-cancer incidence even after adjustment for duration of cigarette smoking, number of cigarettes smoked/day, retinol index and age. Although carotene intake tended to be lower in men who subsequently developed lung cancer than those who did not, mean intakes of retinol, energy and other nutrients were similar in the two groups. This strengthens slightly the view that it is the carotene intake that reduces lung-cancer risk and not some other factor associated with eating fruit and vegetables. The carotene index was significantly inversely related to the 19-yr mortality from lung-cancer but not from other carcinomas, other malignant neoplasms, cardiovascular-renal diseases or other causes. Smoking status was, however, strongly related to risk of death in all categories except malignant neoplasms other than carcinomas.

The authors add a note that these results should be treated with considerable caution since the estimates of carotene and retinol intake were extremely crude.

The metabolic fate of Michler's ketone

Struck, R. F., Shih, T. W., Johnston, T. P., Kirk, M. C. & Hill, D. L. (1981). Metabolism and macromolecular binding of the carcinogen Michler's ketone in rats. *Xenobiotica* **11**, 569.

An early Japanese paper reported that Michler's ketone (4,4'-bis(dimethylamino)benzophenone; MK) which is used in dye and polymer manufacture, produced nodular hyperplasia of the liver and stomach papillomas on prolonged feeding to rats (Kinosita, *Trans. Jap. path. Soc.* 1937, **27**, 665). A subsequent NCI bioassay showed technical-grade MK to cause hepatocellular adenomas and carcinomas when fed to rats and female mice, while male mice developed haemangiosarcomas (*Federal Register* 1979, **44**, 56029; Garner & Cockrell, *Toxic. appl. Pharmac.* 1975, **33**, 172). MK was mutagenic in Drosophila (Rapoport, *C.r. Acad. Sci. U.R.S.S.* 1946, **54**, 65) but not in *Salmonella typhimurium* strains TA98, 100, 1535 and 1538 in the presence of a rat-liver S-9 fraction (Scribner *et al. Cancer Lett.* 1980, **9**, 117). However, when tritiated MK was injected into male rats it bound to liver DNA and RNA, and no decrease in DNA binding was evident after 6 wk (Scribner *et al. loc. cit.*). The present paper confirms that MK binds to nucleic acids, and elucidates its metabolic pathway.

When injected intraperitoneally into rats without prior enzyme induction by phenobarbital (PB), [^{14}C]MK bound irreversibly to the liver proteins. Rats pretreated with PB showed increased binding to liver proteins and also detectable binding to DNA of liver and kidney, to RNA of liver and to proteins of kidney and small and large intestine. At 1, 3 and 24 hr, most radioactivity was found in the intestine and some was present in the liver. After incubation of [^{14}C]MK for 20 min with liver microsomal preparations from PB-pretreated rats, mass spectral analysis revealed that the major microsomal metabolites were asymmetrical di-demethylated and tri-demethylated MK, with lesser amounts of the mono- and tetra-demethylated compounds. Biliary excretion in rats given 10 mg MK/kg by intravenous injection accounted for about 55% of the administered dose in 24 hr, the major products again being the asymmetrical di- and the tri-demethylated compounds, together with *N*-acetylated tetra-demethylated MK. Minor biliary metabolites were tentatively identified as the ring-hydroxylated derivatives of these three compounds and of *N*-acetylated tri-demethylated MK. A rat given 7 mg [^{14}C]MK/kg by stomach tube had after 24 hr eliminated 45% of the ^{14}C in the urine and 23% in the faeces, with 18% remaining in the carcass and none being detected in the expired air. The major metabolite was tentatively identified as a ring-hydroxylated derivative of *N*-acetylated di-demethylated MK, while a minor metabolite lacked the hydroxyl group. Together these two compounds accounted for 68–80% of the urinary radioactivity.

As demethylated MK is structurally similar to benzidine, it is suggested that after demethylation of one or both amino groups and *N*-acetylation by cytosolic enzymes, MK could be *N*-hydroxylated. The resulting metabolite, in the presence of an acyltransferase, could become bound to macromolecules and be responsible for the carcinogenic activity of MK.

Neuropathy from low-level hexane exposure

Ruff, R. L., Petito, C. K. & Acheson, L. S. (1981). Neuropathy associated with chronic low level exposure to n-hexane. *Clin. Toxicol.* **18**, 515.

Polyneuropathy has been caused by exposure to *n*-hexane, generally at concentrations above 500 ppm. However, an atmospheric concentration of only 400 ppm, resulting from a faulty petrol combustion stove, is believed to have caused polyneuritis in three members of a family (Benichou *et al. Bull. Mém. Soc. méd. Hôp. Paris* 1967, **118**, 91; Gaultier *et al. J. Eur. Toxicol.* 1973, **6**, 294), and retinal changes have been reported in Finnish workers exposed to concentrations 'generally' below 500 ppm (*Cited in F.C.T.* 1979, **17**, 686). Rats exposed continuously to 400–600 ppm *n*-hexane for up to 162 days showed clinical and pathological signs of nervous system damage (*ibid* 1977, **15**, 492) and evidence of denerva-

tion was found in mice exposed for 24 hr/day on 6 days/wk for 1 yr to mean concentrations of 272 ppm or more (Miyagaki, *Jap. J. ind Hlth* 1967, **9**, 660). In the last study, a concentration of 99 ppm was without apparent effect. However, even at an 8-hr time-weighted average concentration of only 58 ± 41 (SD) ppm, a group of 14 Japanese workers showed signs suggesting minor functional impairment of the peripheral nervous system (Sanagi *et al. Int. Archs occup. envir. Hlth* 1980, **47**, 69). The TLV currently recommended by ACGIH is 100 ppm, with a 15-min exposure limit of 125 ppm, but it is proposed to reduce the former value to 50 ppm (*TLVs: Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Environment with Intended Changes for 1981*; ACGIH, Cincinnati, OH, 1981).

Ruff *et al.* (cited above) describe a case of peripheral neuropathy from fairly brief exposures to concentrations thought to be no greater than 450 ppm. A 60-yr-old male, who had worked as a janitor for an adhesive-tape manufacturer until 1977, was admitted to hospital in 1979 with a 5-yr history of progressive leg weakness and sensory loss. The company had used *n*-hexane-containing solvents for 6 months in 1971, but had discontinued their use when the first cases of neuropathy were reported in American workers (*Cited in F.C.T.* 1972, **11**, 157). At this time the patient discovered that the solvent containing *n*-hexane was very useful for removing glue stains, and hid a vat for use in cleaning the laboratory table tops. He used the solvent only in the laboratory area, for less than 2 hr/day, and wore gloves to handle the solvent-impregnated rags. In 1973 this practice was discovered by the plant safety manager, but was allowed to continue when five separate analyses revealed that atmospheric concentrations of *n*-hexane did not exceed the TLV, then 500 ppm. Average levels during cleaning were 325 ppm, with a single peak of 450 ppm. No other known neurotoxic chemicals were used.

Distal weakness and numbness of the legs were first evident in 1974 and progressed, until in 1977 the patient could no longer walk unaided. Electromyography indicated denervation in distal leg muscles, with reduced conduction velocity in the motor nerves, and sensory nerve responses were absent in distal areas of both arms and legs. Two years after his retirement only slight improvement was evident, and a sural nerve biopsy showed axonal changes typical of *n*-hexane neuropathy. The slow onset of the condition was attributed to the low level of exposure and its intermittent nature (less than 10 hr/wk in total).

[This case provides further confirmation that man may be more sensitive than rats or mice to *n*-hexane neuropathy. In a study that has yet to be reported in full, no pathological evidence of neurotoxicity was found in rats exposed for 6 hr/day, 5 days/wk for 90 days to as much as 10,000 ppm *n*-hexane (*Chemical Industry Institute of Toxicology 1980 Annual Report and Scientific Review: Science in the Public Interest: CIIT Activities* 1981 **1**(3), 3) and in the other animal studies cited above continuous exposure was required to elicit neurotoxic effects.]

Non-mutagenicity of toluene and xylenes

Bos, R. P., Brouns, R. M. E., van Doorn, R., Theuws, J. L. G. & Henderson, P. Th. (1981). Non-mutagenicity of toluene, *o*-, *m*- and *p*-xylene, *o*-methylbenzylalcohol and *o*-methylbenzylsulfate in the Ames assay. *Mutation Res.* **88**, 273.

In 1977 the Interagency Testing Committee recommended further testing of xylenes and toluene, for effects including mutagenicity in the former case and carcinogenicity in the latter (*Federal Register* 1977, **42**, 55026). A 2-yr rat test on toluene at inhaled concentrations of 30–300 ppm has since demonstrated no evidence of carcinogenicity or other adverse effects (*Chemical Industry Institute of Toxicology 1980 Annual Report and Scientific Review: Science in the Public Interest*) and neither xylene nor toluene was found to affect the number of sister chromatid exchanges or chromosome aberrations in human lymphocytes *in vitro* (Gerner-Smidt & Friedrich, *Mutation Res.* 1978, **58**, 313). *In vivo*, there was a similar lack of effect on the lymphocytes of 32 male rotogravure workers with daily exposure to toluene (Mäki-Paakkanen *et al. J. Toxicol. envir. Hlth* 1980, **6**, 775), confirming previous observations (*Cited in F.C.T.* 1972, **10**, 271).

Despite these reassuring findings, the metabolic pathways of both compounds give theoretical grounds for concern. Cresols and dimethylphenols have been identified as minor urinary metabolites of toluene and xylenes respectively, in both the rat (*ibid* 1970, **9**, 429) and man (Angerer, *Int. Archs occup. envir. Hlth* 1979, **43**, 63; Pfäffli *et al. Scand. J. Work envir. Hlth* 1979, **5**, 286; Sedivec & Flek, *Int. Archs occup. envir. Hlth* 1976, **37**, 205). These phenolic compounds are thought to result from rearrangement of arene oxide intermediates, reactive nucleophiles that may function as proximate carcinogens (Jerina & Daly, *Science, N.Y.* 1974, **185**, 573; Jerina *et al. Proc. natn. Acad. Sci. U.S.A.* 1971, **68**, 2545). Evidence has also been obtained in the rat that toluene and xylenes undergo side-chain metabolism to other reactive intermediates, which are conjugated with glutathione and excreted as mercapturic acids; these urinary products accounted for about 1% or less of the dose of toluene and *p*- and *m*-xylenes, but for as much as 10–21% of *o*-xylene (van Doorn *et al. Archs Toxicol.* 1980, **43**, 293). Experiments with metabolic inhibitors suggested that sequential side-chain oxidation and sulphation preceded glutathione conjugation (*idem, Toxicology Lett.* 1980, Special Issue no. 1, p. 219).

To investigate whether these reactive intermediates may function as mutagens, Bos *et al.* (cited above) subjected toluene and *o*-, *m*- and *p*-xylenes as well as *o*-methylbenzyl alcohol and *o*-methylbenzyl sulphate (the products of side-chain oxidation and sulphation, respectively, of *o*-xylene) to Ames tests, using *Salmonella typhimurium* strains TA1535, 1537, 1538, 98 and 100. However, both in the absence and presence of S-9 mix derived from untreated or Aroclor 1254-induced rats, uniformly negative results were obtained. Tests for DNA-excision repair in suspensions of freshly isolated rat hepatocytes, using concentrations of 10^{-7}–10^{-3} M, also failed to provide evidence of genotoxicity.

[When considered in conjunction with the earlier negative mutagenicity and carcinogenicity results, these findings suggest that the formation of reactive

intermediates from toluene and xylenes may be of little toxicological consequence.]

Acrylonitrile epidemiology study

Werner, J. B. & Carter, J. T. (1981). Mortality of United Kingdom acrylonitrile polymerisation workers. *Br. J. ind. Med.* **38,** 247.

Although acrylonitrile (ACN) is widely used in industry, particularly for the manufacture of rubber and synthetic fibres, its carcinogenic and mutagenic potential in man still remains to be clarified. ACN has been shown to be mutagenic in some strains of *Salmonella typhimurium* and *Escherichia coli* (*Cited in F.C.T.* 1979, **17,** 179; *ibid* 1979, **17,** 554) and can cause transformation of, and damage to DNA in, Syrian golden hamster embryo cells (*ibid* 1980, **18,** 442). However, no evidence of an increased incidence of chromosomal aberrations was noted in a study of the lymphocytes of workers exposed to ACN (*ibid* 1979, **17,** 554). Suggestions that ACN may have a carcinogenic effect in man have stemmed in part from rat studies involving chronic inhalation and oral exposure (*Food Chemical News* 1981, **22** (45), 19; Maltoni *et al. Medna Lav.* 1977, **68,** 401) and from an epidemiology study suggesting that workers exposed to ACN suffered an excess risk of cancer, particularly lung cancer (O'Berg, *J. occup. Med.* 1980, **22,** 245). However, two other epidemiological studies (Kiesselbach *et al. Zentbl. ArbMed. ArbSchutz* 1979, **10,** 257; Thiess *et al. ibid* 1980, **30,** 259) yielded equivocal results on the effects of ACN exposure in man. The present study was therefore set up to investigate whether a carcinogenic risk existed for workers exposed to ACN in the United Kingdom.

The mortality of 1111 men who worked in six factories on the polymerization of ACN and the spinning of acrylic fibre from 1950 to 1968 was surveyed up to the end of 1978. There were fewer deaths than expected (on the basis of standard age-specific mortality rates for England and Wales) amongst the 177 workers who had been exposed to ACN for less than 1 yr, both overall and for each cause of death excepting suicide. Similarly, in those men (934) exposed to ACN for a year or more, the total number of deaths was found to be smaller than expected. However, slightly more deaths from cancer were observed than were expected (due mainly to cancers of the lung, stomach, colon and brain), although this increase in all cancer deaths did not appear to be statistically significant. However, the increased incidence of deaths from stomach cancer, in the group as a whole and in the men aged 55–64, was found to be of statistical significance, as was the increased risk of death from lung cancer in those aged 15–44. Fewer deaths were observed than were expected for diseases of the circulatory and respiratory systems, although there was an increased incidence (not statistically significant) of deaths from bronchitis, particularly in the 55–64 age group.

The higher death rate from lung cancer was investigated further, since it occurred in relatively young men (three workers in the 15–44 age group). However, no consistent difference was found between the duration of ACN exposure in these three cases and in matched controls (all those in the study cohort born in the same year and outliving the lung-cancer victim). The authors point out that information on the smoking habits of the participants would have been particularly useful in interpreting the findings on lung cancer. Nevertheless, no statistically significant excess of deaths from circulatory or respiratory diseases (which are also associated with smoking) was noted amongst the workers, suggesting that the study population did not have greatly excessive smoking habits.

It was noted that all those in the study population who died from stomach cancer worked in factories in Wales, where the mortality rate from stomach cancer during the period covered by the study was well above the England and Wales average. This suggests that the observed excessive incidence of stomach cancer may have been due to regional rather than occupational factors.

The authors point out that the methods of analyses they used were greatly influenced by the brevity of the follow-up period and the resulting small number of deaths expected in the study population. An extension of the study to include a longer follow-up period would obviously be useful. They also emphasize the difficulties in choosing a suitable standard population to provide rates for calculating numbers of expected deaths, since these are influenced by the regional, social and employment status of individuals. Summing up, the authors state that the results obtained are inconclusive and neither add to, nor detract from, existing suspicions that ACN is a human carcinogen. However, when the observations are considered in the light of evidence from other studies, a need is suggested for the continuing surveillance of exposed populations.

[No data concerning actual levels of ACN exposure were available. Such measurements would obviously be of use in interpreting the reported observations.]

Occupational bladder cancer—more questions than answers

Checkoway, H., Smith A. H., McMichael, A. J., Jones, F. S., Monson, R. R. & Tyroler, H. A. (1981). A case-control study of bladder cancer in the United States rubber and tyre industry. *Br. J. ind. Med.* **38,** 240.

The link between occupational exposure to certain chemicals used in the rubber and related industries and bladder cancer is well known (*Cited in F.C.T.* 1970, **8,** 210) but the source of the carcinogenic activity has never been fully established. Recognition that compounds such as β-naphthylamine, 4-aminobiphenyl and benzidine are bladder carcinogens eventually led to the banning or extremely strict control of their use in rubber in the UK in 1967, although in practice industrial use had been largely discontinued many years earlier because of increasing concern (Occupational Cancer in the Renal Tract. Technical Data Note 3 (Rev). Department of Employment, HM Factory Inspectorate, 1976). During the years relevant to the American study discussed here, the use of β-naphthylamine, 4-aminobiphenyl and benzidine as antioxidants in rubber had been superseded by that of another aromatic amine, phenyl-β-naphthylamine, although this is now also listed by the ACGIH as a suspected human carcinogen (*TLVs: Threshold Limit*

Values for Chemical Substances and Physical Agents in the Workroom Environment with Intended Changes for 1978; ACGIH, Cincinatti, OH, 1978).

Checkoway *et al.* (cited above) describe a retrospective survey of workers employed in five rubber and tyre factories in the USA between 1910 and 1975, designed to determine whether the development of bladder cancer was associated with any particular job or work area within the plants. It seems from their results that men employed in the milling and calender-operation sectors, which entail potential exposure to volatilized reaction products from heated rubber, were more likely to develop bladder tumours than were their colleagues who had been involved in other stages of the manufacturing processes.

All of the subjects were hourly paid workers from one of five large rubber companies (A–E) in the same area of Ohio. Cases of bladder cancer amongst former male employees were identified either by searching the local hospital records for the period 1954–1974, or by screening death certificates between 1964 and 1973 for employees at companies A, D and E, between 1950 and 1973 for company C and between 1940 and 1974 for company B. Each bladder cancer case thus identified was then matched with two controls by company, race and year of birth (±2 yr); one of the controls was also matched by year of hire (±2 yr) and duration of employment (±2 yr). The lifetime work histories of all the 220 cases and their controls were examined and each subject was allotted to one (or more) of 21 mutually exclusive 'occupational title groups' (OTGs) for the purpose of classifying exposure. Thus it was possible to estimate the relative risks for bladder cancer associated with each OTG using standard statistical techniques.

Comparison of cases and controls not matched by year of hire and age of hire showed no differences for those variables, suggesting that age and calender year of initial exposure to the industry were not risk determinants. The two control groups were therefore combined for subsequent calculations. It was found that bladder cancer cases were more likely than controls to have worked for at least 1 month in milling and calender-operation jobs. The odds ratios for these jobs (1·91 and 2·21, respectively) were the only two from the 21 OTGs that were substantially greater than the expected value of 1·00. Relative risk estimates for both of these OTGs increased with length of exposure, but no apparent association was found between age or year of first exposure and excess risk of bladder cancer. A weaker and not statistically significant association between work on the final inspection of tyres and bladder cancer risk was observed. In this case the relative risk did not increase with increasing duration of exposure.

Examination of the relative risks associated with these jobs company by company produced some conflicting data but the results from company C, which was the largest plant and the one with the greatest number of exposed subjects, were similar to the results from the total set.

A good epidemiology study is notoriously difficult to achieve. Aware of certain deficiencies, the authors stress that this was only an exploratory study. The lack of detailed qualitative and quantitative information on exposures associated with different jobs, compounded by movement between jobs and also potential confounding by factors such as cigarette smoking, necessitate guarded conclusions. In addition, loss of data on cases of bladder cancer amongst former employees who moved out of the area may have led to a dilution of any trends in this study.

The authors speculate briefly on the source of the cancer problem, suggesting that aromatic amines generated by the use of chemicals such as *N*-nitrosodiphenylamine, diphenylamine and aldol-α-, phenyl-α- and phenyl-β-naphthylamines could be the offending bladder carcinogens. However, as is frequently the case in toxicology, this study produces more questions than answers.

Mutagenic nitropyrenes

Mermelstein, R., Kiriazides, D. K., Butler, M., McCoy, E. C. & Rosenkranz, H. S. (1981). The extraordinary mutagenicity of nitropyrenes in bacteria. *Mutation Res.* **89,** 187.

Mutagenic nitroarenes can be formed in various environmental samples as a result of the nitration of polycyclic aromatic hydrocarbons (PAHs), which originate from incomplete combustion processes (Pitts, *Phil. Trans. R. Soc. A* 1979, **290,** 551; Pitts *et al. Science, N.Y.* 1978, **202,** 515; Rosenkranz *et al. ibid* 1980, **209,** 1039). Furthermore, mutagenic nitropyrenes have been identified as trace impurities in carbon black and selected xerographic toners (Rosenkranz *et al. loc. cit.*). Mermelstein *et al.* (cited above) examined the mutagenic properties of six nitrated derivatives of pyrene—1-nitropyrene (I), 1,3-dinitropyrene (II), 1,6-dinitropyrene (III), 1,8-dinitropyrene (IV), 1,3,6-trinitropyrene (V) and 1,3,6,8-tetranitropyrene (VI). These compounds were believed to be at least 99% pure.

All six exhibited very potent mutagenic activity at concentrations up to 1 μg/plate using *Salmonella typhimurium* strains TA98, 1538 and 1537, which are susceptible to frameshift mutations. No microsomal activation system was required to produce this effect. Indeed it has previously been reported that the addition of such activation systems decreases the mutagenicity of nitropyrenes (Rosenkranz *et al. loc cit.*). In contrast, there was little mutagenic activity in strain TA1535 and in *Escherichia coli* WP2 *uvr* A, which are used to detect base substitution mutations. Activity also tended to be low in strains TA1978 and 1977, but was relatively high in TA100. Unexpectedly, the mutagenic activity of three of the nitropyrenes (III, IV and V) was not significantly lower when they were tested in strain TA98NR, the nitroreductase-deficient TA98 derivative that has been shown to lack the enzyme that activates nitrofurans, nitronaphthalenes and nitrofluorenes to mutagens (Rosenkranz & Mermelstein, in *The Predictive Value of Short-term Screening Tests in Carcinogenicity Evaluation*, p. 5. Edited by G. M. Williams *et al.* Elsevier/North-Holland Medical Press, Amsterdam, 1980). These results suggest either that the nitropyrenes are direct-acting and do not require reductive conversion to the corresponding hydroxylamines, or, more probably, that if a reductive process does occur, it can be performed by

an enzyme other than the 'classical' nitroreductase (the one that acts on nitrofurans and other simple nitrated PAHs).

Frameshift mutation is often due to the intercalation of a chemical between DNA base pairs. However, the authors suggest that the potent activity exhibited by the nitropyrenes (or their metabolites) is more consistent with the formation of adducts between DNA and the chemicals. This view is supported by the decreased mutagenicity observed with strains TA1977 and 1978 which are *uvrB*$^-$ analogues of TA1537 and 1538, respectively. These analogue strains respond in an undiminished fashion to chemicals that cause frameshift mutation as a result of intercalation, but show a greatly decreased mutagenic response when exposed to chemicals that form adducts recognized by UV endonuclease, the product of the *uvrB*$^+$ gene (Rosenkranz & Mermelstein, *loc. cit.*).

The authors conclude that nitropyrenes are potent mutagens in some strains of *S. typhimurium*, causing frameshift mutations by the formation of adducts between DNA and nitropyrene metabolites. They also postulate that the penultimate intermediates for mutagenic activity (hydroxylamines) are not obtained in all instances by reduction of the nitro function by the 'classical' nitroreductase but by another nitroreductase which appears to be specific for higher nitrated PAHs.

[The authors do not record having used 'positive' and 'negative' controls in this study. Several of the suggestions put forward are based partly on unpublished observations, making appraisal somewhat difficult. Some of these unpublished results are reported to show that the 'new' nitroreductases may be unique to bacteria.]

Filtering out the coronaries?

Castelli, W. P., Garrison, R. J., Dawber, T. R., McNamara, P. M., Feinleib, M. & Kannel, W. B. (1981). The filter cigarette and coronary heart disease: the Framingham study. *Lancet* **II**, 109.

The smoker receives regular advice on his particular habit from people in all walks of life. Of these, politicians and clinicians probably contribute most strongly to any observed alteration in smoking behaviour. Certainly, the introduction of filtered cigarettes in 1950 led many people to change brands perhaps on the assumption that this might reduce the risk of developing cancer, coronary heart disease or pulmonary disease.

However, these assumptions can rarely be tested. The paper cited above discusses one test that has been made using the cohort of people taking part in the long-running study at the National Heart, Lung, and Blood Institute, Framingham, Massachusetts. Regular examinations have been carried out on this group every 2 yr since 1948 and the results presented are from data collected at the first and at the seventh to fourteenth examinations. Examination 12 was the most recent for which smoking data were available. It was impossible to analyse the data for the women involved in the study since only the youngest smoked at the time of the seventh examination, carried out in 1963–1964. The 1605 men who smoked at the seventh examination were divided into two groups according to age (above or below 55).

A greater proportion of the younger than of the older group smoked cigarettes (60 and 42%, respectively) but the proportion of smokers using filters was similar (58 and 57%, respectively). The filter smokers typically had slightly lower smoking exposure prior to examination 7 than did smokers of non-filter cigarettes but otherwise there were few differences between the two groups in overall smoking behaviour.

The smoking data were compared with the rates of occurrence of myocardial infarction, coronary heart disease and death from coronary heart disease over the 14-yr period (examinations 7–14). No clear trends were observed in the older group but in the younger, the non-smokers had the lowest rates of heart disease and the filter-cigarette smokers the highest. However, no statistically significant differences were observed. These findings were unchanged after adjustment for age, systolic blood pressure and serum cholesterol levels.

Filter-cigarette smokers have been observed to have a lower cancer mortality, a finding presumably due to the reduction in tar exposure (Bross & Gibson, *Am. J. publ. Hlth* 1968, **58**, 1396; Wynder & Stellman, *J. natn. Cancer Inst.* 1979, **62**, 471). Two other components of cigarette smoke, nicotine and carbon monoxide, have been studied in connection with coronary heart disease. Data on trends in the nicotine content of cigarettes during the study period indicated that the smokers of filter cigarettes had lower nicotine exposure, and therefore it appears that nicotine lowering does not have much effect on the risk of coronary heart disease in smokers of filter cigarettes. On the other hand carbon monoxide has previously been shown to be associated with atherosclerosis, a common cause of heart failure (*Cited in F.C.T.* 1974, **12**, 150), and carbon monoxide levels may actually be increased by some filters, particularly unperforated ones. The possibility that the slightly higher rates of coronary heart disease in filter-cigarette smokers was due to a selection of such cigarettes by men who had had subclinical 'warning' of the disease seems unlikely, since most of the men had been smoking filter cigarettes for a number of years before examination 7. Suspicion about the benefits of filters is also raised by the fact that the men in the filter-cigarette group had had more favourable smoking histories prior to examination 7.

This study indicates that the original philosophy that many of the risks associated with smoking would be reduced by the use of filters may well be erroneous, at least as far as coronary heart disease is concerned.

Amongst the older group of men, less than one third continued to smoke cigarettes until examination 12, and those who continued to smoke had reduced their daily consumption. Significant numbers of men in the younger age group had also given up smoking. A considerable number of men (11–42%) were lost to follow-up at examination 12.

In commenting on the study described above, Lee (*Lancet* 1981, **II**, 642) cites conflicting findings from a recent review (Lee & Garfinkel, *J. Epidem. commun. Hlth* 1981, **35**, 16) of available evidence on mortality

and type of cigarette smoked, which suggested that coronary heart disease mortality was 10–20% lower in smokers of filter cigarettes than in smokers of plain cigarettes. Lee points out that the numbers involved in the Framingham study put severe limitations on the conclusions that can be drawn from the data. The studies considered in his review involved much larger numbers of deaths. However Castelli (*Lancet* 1981, **II**, 642) replies that the Framingham study did allow consideration of other potentially confounding variables such as diet and exercise.]

Carcinogenicity confounded

Salsburg, D. (1980). The effects of lifetime feeding studies on patterns of senile lesions in mice and rats. *Drug Chem. Toxicol.* **3**(1), 1.

The evaluation of the long-term toxicity of compounds in man is beset with problems, not the least of which is the interpretation of data that arise from animal bioassays. The paper cited here questions the validity of conventional methods of analysis.

It is argued that the statistical models traditionally applied to chronic toxicity studies to not take account of essential biological considerations. Current methods of analysis are based on a procedure in which, taking each lesion in turn, all of the animals are assigned to one of only two categories—those that develop the lesion and those that do not. For comparison between treated groups and controls, the incidence of one lesion is treated as independent of that of any other. Salsburg suggests that this ignores a fundamental toxicological principle. He believes that consideration should be given to the premise that a biologically active compound will actually produce a dose-related shift in the pattern of lesions. In subchronic tests, that shift is easily followed, but lifetime studies are more difficult to interpret since the pattern of lesions in old control animals is itself complex.

More effective analysis should begin with a search for patterns of lesions that differ between treatment groups. If independent clusters of lesions are located, it is possible to decide whether the treatment induces a shift of animals from one cluster to another. Using carcinogenicity data generated at the Huntingdon Research Centre on xylitol, sorbitol and sucrose, Salsburg illustrates his point by applying a multivariate analysis to data previously analysed by conventional univariate methods. The method of multivariate analysis used allows the patterns of statistical correlations among various lesions to cluster animals into groups that appear to have common syndromes and, finally, the relationships between the clusters and treatment are examined. One important point arises from this reanalysis. It seems to Salsburg too simplistic to report, without qualification, that xylitol is a carcinogen in the rat. Although xylitol increases, in a dose-related manner, the incidence of adrenal phaeochromocytomas, it can be shown from the same experimental data that it also decreases, again in a clearly defined dose-related manner, the incidence of some other types of tumour, such as cutaneous fibrosarcoma. Other (fairly convincing) reasons are offered in support of the multivariate approach.

Salsburg concludes that some fresh thought should be given either to the way in which carcinogenicity bioassays are conducted at present or to the evaluation of the data that conventional bioassays generate. Because of the complexities of the pathology found in experiments involving animals in advanced old age, some consideration could be given to reducing the length of studies so that the treated animals can be compared with a relatively uncomplicated baseline of lesions in the controls. If it is unacceptable to turn the clock back—carcinogenicity studies conducted two decades or so ago often did not exceed 12 months—then more complex methods of data analysis must be used.

[This is bad news for non-statistically-minded toxicologists. Multivariate analysis, if it does become the acceptable way to proceed, will bring down the curtain on an era of accessible carcinogenicity verdicts. The days when a summary of data from a study would allow an 'expert' to evaluate potential carcinogenicity may well be numbered.]

Screening out the irritations in life

Burton, A. B. G., York M. & Lawrence, R. S. (1981). The *in vitro* assessment of severe eye irritants. *Fd Cosmet. Toxicol.* **19**, 471.

The urgent—some would say vital—search for more humane methods of testing new or everyday chemicals for their likely toxicological hazards has encouraged the development of new approaches to applied toxicological research. One area of activity has been in methods of assessing potential eye irritancy to replace the successful but often painful Draize test (Draize *et al. J. Pharmac. exp. Ther.* 1944, **82**, 377).

A method that has now been devised involves the careful removal of the eyes from newly killed New Zealand white rabbits previously confirmed as healthy and free from ocular abnormalities. Each eye is held by means of a perspex clamp in a temperature-controlled 'superfusion' chamber and isotonic saline is dripped onto the cornea at the limbus. The temperature is maintained, by means of a water jacket, at 32 ± 1 C, the pre-ocular temperature in the rabbit. The test substance is applied to the eye instead of the saline for a set time (usually 10 sec) and is then removed by rinsing, after which the saline superfusion is continued. The effects are assessed for up to 4 hr after this treatment using a slit-lamp biomicroscope, the walls of the compartments being of black perspex to facilitate these observations. Corneal thickness is also measured.

A series of chemicals were tested in this way, including known severe irritants such as sodium hydroxide and formaldehyde, moderate irritants such as allyl alcohol and acetone, and slightly or negligibly irritating compounds such as glycerine and propylene glycol. The results were generally comparable with those of a series of *in vivo* tests reported by Carpenter & Smyth (*Am. J. Ophthal.* 1946, **29**, 1363), particularly for the severely irritating chemicals, which must cause the greatest pain when tested in live animals. There were, however, some notable differences. Toluene, for example, was moderately to severely irritating (grade 7) to the rabbit eye in the *in vivo* test undertaken by

Carpenter & Smyth *loc. cit.*) yet produced only a negligible or slight reaction in the *in vitro* test. Ethanol gave the opposite result, with a moderate *in vitro* reaction compared with a grade of only 3 in the reported Draize test.

These differences may be no more than the normal variability between Draize tests undertaken at different laboratories, a variability that has previously given rise to concern (*Cited in F.C.T.* 1974, **12,** 589). However, they may also result from the ability of the live animal to recover following this type of toxicological insult, or they may indicate a particular susceptibility to the compound which is not just a feature of the reaction of the eye itself. Such cases may well justify the continuing use of the Draize method, but only for slight or moderate irritants; severe irritants could be eliminated by preliminary screening using an *in vitro* procedure such as that described in this paper.

Fd Chem. Toxic. Vol. 20, p. 493
Pergamon Press Ltd 1982. Printed in Great Britain

LETTERS TO THE EDITOR

IMPACT OF AIR-LEAD ON BLOOD-LEAD IN MAN

Sir,—In calculating the predicted blood-lead concentrations given in Fig. 2 of our paper "The impact of air-lead on blood-lead in man—A critique of the recent literature" (*Fd Cosmet. Toxicol.* 1981, **19**, 631) from the two lead disposition models considered, it was necessary to assign to the blood some but not all of the lead contained in the central compartment. In making this assignment, we used the relationship—blood-lead concentration in μg/dl = 45 times the amount of lead (in mg) in the central compartment—given by Ashford *et al.* (in The Effects of OSHA Medical Removal Protection on Labor Costs of Selected Lead Industries: Report of the Center for Policy Alternatives, Massachusetts Institute of Technology, Cambridge, MA, 1977; under Contract No. 172646 from the US Department of Labor). This relationship was based on the Bernard model. Dr M. B. Rabinowitz has subsequently pointed out to us that a better relationship to use in conjunction with the Rabinowitz model would be one based on compartment sizes, as estimated by Rabinowitz *et al.* (*J. clin. Invest.* 1976, **58**, 260) by assuming concentration throughout the central compartment to be equal to blood-lead concentration. The effect of such an adjustment would be to bring the curve for the Rabinowitz model closely into line with that for the Bernard model in Fig. 2, and to reduce proportionately the values given for blood-lead for the Rabinowitz model in Table 2. Thus, the result using the same relationship to assign central-compartment lead to blood-lead for both models is to exaggerate the discrepancy between predicted and observed blood-lead in the case of the Rabinowitz model.

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Fd Chem. Toxic. Vol. 20, pp. 494–495
Pergamon Press Ltd 1982. Printed in Great Britain

COMMENT ON GUINEA-PIG TEST METHODS

Sir,—I read with interest a recent article by Marzulli & Maguire (*Fd Chem. Toxic.* 1982, **20**, 67) in which they used statistical analysis to compare the results of various methods to test skin sensitization in guinea-pigs with published information about "predictive" human tests. One of the methods they purported to use was the Buehler method and they cited some references that describe this procedure (Buehler, *Archs Derm.* 1965, **91**, 171; Ritz & Buehler, in *Current Concepts in Cutaneous Toxicity*. Edited by V. A. Drill & P. Lazar, p. 25. Academic Press, New York, 1980). These references clearly specify and describe why an occlusive patch site is essential to the proper performance of the test and describe in detail a method of restraint that can guarantee this occlusivity. It has been known since the time of the original publication that "wrapping" guinea-pigs for this purpose is inadequate and can lead to failure. The other criteria essential to the successful performance of the test, including procedures to select dose and vehicle, are discussed in the paper by Ritz & Buehler (*loc. cit.*). Procedures that do not meet these criteria should not be represented as the Buehler method, particularly when the results are used to discourage its use.

It is important that all toxicologists and immunologists understand the importance of occlusivity. Restrainers to achieve this are available commerically. Without restraint the procedure is inadequate. As a process to validate the methodology, we suggest that laboratory personnel be able to induce sensitization in guinea-pigs with a single induction dose of 0·3% 2,4-dinitro-1-chlorobenzene (DNCB) in ethanol. At challenge most or all animals should respond positively to 0·2% DNCB (in acetone) and about half should respond to 0·02%. If the procedure described by Ritz & Buehler (*loc. cit.*) is followed, success is guaranteed.

Readers should also know that using the Buehler method, we have readily sensitized guinea-pigs to 5% formalin (2% formaldehyde in water). These data were reported in 1965 (Buehler, *loc. cit.*) and have been repeated more recently during some comparative studies on sensitization with other preservatives (E. A. Newmann, unpublished data, 1982). We have also successfully sensitized guinea-pigs to hydroxycitronellal (H. L. Ritz, unpublished data, 1982): although 10% hydroxycitronellal in 80% ethanol did not induce sensitization, 30% did so readily. Whether or not ethanol is truly a sensitizer or produces hyper-reactivity by some other mechanism is still an open question; however, the original observations of sensitization in guinea-pigs and man (Stotts & Ely, *J. invest. Derm.* 1977, **69**, 219) were after the use of 80 and 50% aqueous ethanol, respectively. We have not had any experience with the other materials mentioned by Marzulli & Maguire (*loc. cit.*), but for the substances we have tested the correlation between the methods we use is very good.

One other comment appears to be appropriate. A prospective approach for the safety assessment of any chemical to be used in consumer products requires more than just the detection of a biological property. Whether or not the biological effect will be expressed adversely in man depends on the many factors (dose, exposure conditions, time of exposure etc.) that can be anticipated under intended conditions of use or as a result of accidental exposures. A more realistic approach, specifically addressed to potential sensitizers, is to be presented this July at the Toxicology Forum in Aspen, CO.

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Sir,—We and others (Magnusson & Kligman, in *Identification of Contact Allergens*. C. C. Thomas, Springfield, IL, 1979) have found that wrapping the sensitization (test) site with an occlusive dressing is a perfectly satisfactory way of occluding the site. If this is properly done, the dressing does not slip or slide. Further, such dressings are regularly used to provide occlusive applications in patients and in human skin testing.

In our study (*Fd Chem. Toxic.* 1982, **20**, 67), concentrations of test compounds and their vehicles were chosen so as to coincide with formulations that had already been tested in humans. This allowed us to make comparisons between human testing and testing in guinea-pigs; this was a major point of our study.

In should be noted that Dr Buehler's 1965 report dealing with the sensitization of guinea-pigs to 5% formalin indicated that there was a detergent (alkylbenzene sulphonate; ABS) in the vehicle (Buehler, *Archs Derm.* 1965, **91**, 171). This detergent would be expected to enhance the immunogenicity of the test material. We did not use ABS or other detergent in our 5%-formalin test solution, so comparisons cannot be made between Dr Buehler's results and our own. Likewise his results with hydroxycitronellal in 80% ethanol and our results using hydroxycitronellal in a petrolatum vehicle are incommensurable. It is interesting that Dr Buehler found that 10% hydroxycitronellal in 80% ethanol

did not induce sensitization, since he has found that 80% ethanol induces some sort of skin sensitization in guinea-pigs.

Our findings are consistent with the conclusion that the Buehler method will identify moderate and most weak sensitizers but may miss the very weak sensitizers, i.e. those giving a sensitization rate of 5% or less in human testing. Indeed, experiments have been published comparing the allergenicity of compounds in guinea-pigs (using the Buehler technique) and in humans (e.g. Griffith & Buehler, in *Cutaneous Toxicity*. Edited by V. A. Drill & P. Lazar. pp. 155–173. Table 9. Academic Press, New York, 1977) and the findings were very similar to ours. Adjuvant techniques are more sensitive than the Buehler technique and are more reliable for the detection of weak and very weak contact sensitizers.

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Ed Chem. Toxic. Vol. 20, p. 496
Pergamon Press Ltd 1982. Printed in Great Britain

APPARENT IMMUNOTOXIC RESPONSE TO PHENOLIC COMPOUNDS

Sir,—We have had occasion to study several patients who, in the course of their work as laboratory technicians, allergists and the like, have become severely disabled as a consequence of overexposure to phenol present in phenolic germicidal antiseptics (hexachlorophene) or phenylated allergenic extracts. They appear to have suffered an immunotoxic response to phenolics, leading, we believe, to a modification of their immune response. Consequently, whenever they encounter aromatics in the atmosphere at concentrations as low as 1 or 2 ppb*, they undergo adverse neurological responses. The medical management of these patients is extremely complex and challenging.

Our clinical studies to date indicate marked T-cell suppression in these patients, and laboratory studies in the animal model of this condition showed that T-cell suppression occurred (LaVia & LaVia, in *Inadvertant Modification of the Immune Response*; Proceedings of the 4th FDA Symposium. HHS Publn no. (FDA) 80-1074, 1978, p. 148).

As a result of our conversations with immunologists in different parts of the country, we have reason to believe that there may be a very large number of these patients who have not yet been completely and accurately diagnosed. Therefore, we have developed a registry of patients with this illness. We have the facilities and the interest to accumulate clinical and laboratory information on these patients and to perform immunological studies on their serum.

We would appreciate hearing from your readers regarding any cases of patients fitting the above description who were exposed to phenolic chemicals in their work or home environments.

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*b = 10^9.

Fd Chem. Toxic. Vol. 20, p. 497
Pergamon Press Ltd 1982. Printed in Great Britain

MEETING ANNOUNCEMENTS

BTS AUTUMN MEETING

The next meeting of the British Toxicology Society entitled "From Toxin to Therapeutic Substance" will be held at the University of York on 23–24 September 1982. Further details may be obtained from Dr S. M. A. Doak, Shell Toxicology Laboratory (Tunstall), Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG (tel. 0795-2444, ext. 344 or 650).

FOOD CHEMISTRY SYMPOSIUM

A symposium entitled "The Chemistry of Physiologically Active Compounds in Foods and Feedingstuffs" organized by the Food Chemistry Group of the Royal Society of Chemistry is to be held in London on 26 October 1982. Among the topics covered will be, glycoalkaloids in potatoes, glucosinolates in cruciferous vegetables and oilseeds, nitrosamines in beverages, mycotoxins in dairy products, haemagglutinins in beans, food allergies associated with cereal products, and food toxicology. Further details and application forms are available from Mr H. L. Bennister, 174 Beverley Drive, Edgware, Middlesex HA8 5ND (tel. 01-952 4154).

INORGANIC AND NUTRITIONAL ASPECTS OF CANCER

A conference entitled "Inorganic and Nutritional Aspects of Cancer and Other Diseases" organized by The International Association of Bioinorganic Scientists is to be held at La Jolla Village Inn, La Jolla, CA on 10–13 November 1982. Further information may be obtained from Dr G. N. Schrauzer, Department of Chemistry, University of California, San Diego, Revelle College, La Jolla, CA 92093.

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FORTHCOMING PAPERS

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

The metabolic disposition of ^{14}C-labelled Ponceau 4R in the rat, mouse and guinea-pig. By J. C. Phillips, C. S. Bex and I. F. Gaunt.

Influence of gallic acid esters on drug-metabolizing enzymes of rat liver. By M. Depner, G. F. Kahl and R. Kahl.

Subchronic studies in rats fed gum karaya. By P. J. Y. Taupin and D. M. W. Anderson.

Short-term toxicity of 2-phenylpropan-1-ol (hydratropic alcohol) in rats. By I. F. Gaunt, M. G. Wright and R. Cottrell.

Mutagenicity screening of popular Thai species. By M. Ungsurungsie, O. Suthienkul and C. Paovalo.

Formation of mutagens in boiled pork extract. By J.-Y. Lin, H. Lee and H.-I. Huang.

Inhibition of protein pyrolysate mutagenicity by retinol (vitamin A). By L. Busk, U. G. Ahlborg and L. Albanus.

Toxicological evaluation of compounds found in food using rat renal explants. By R. C. Braunberg, O. O. Gantt and L. Friedman.

High incidence of angiosarcomas in brown-fat tissue and livers of mice fed sterigmatocystin. By M. Enomoto, J. Hatanaka, S. Igarashi, H. I. Uwanuma, S. Asaoka, A. Iyatomi, S. Kuyama, T. Harada and T. Hamasaki.

Bacterial mutagenicity studies on chloroform *in vitro*. By N. J. Van Abbé, T. J. Green, E. Jones, M. Richold and F. J. C. Roe.

Evaluation of the cutaneous-irritation potential of 56 compounds. By J. P. Guillot, J. F. Gonnet, C. Clement, L. Caillard and R. Truhaut.

Evaluation of the ocular-irritation potential of 56 compounds. By J. P. Guillot, J. F. Gonnet, C. Clement, L. Caillard and R. Truhaut.

N-Nitroso-*N*-methyldodecylamine and *N*-nitroso-*N*-methyltetradecylamine in household dishwashing liquids. By J. B. Morrison and S. S. Hecht.

Quantitative analysis of catechol and 4-methylcatechol in human urine. By S. Carmella, E. J. LaVoie and S. S. Hecht.

Enteral absorption and biotransformation of the food additive octyl gallate in the rat. By G. Koss and W. Koransky. (Short Paper)

Nitrosation of sarcosine, proline and 4-hydroxyproline by exposure to nitrogen oxides. By C. Janzowski, R. Klein, R. Preussmann and G. Eisenbrand. (Short Paper)

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Aims and Scope

The Journal publishes original papers and reviews relating to the interests of the British Industrial Biological Research Association. This is a wide-ranging field covering all aspects of toxicology but with particular reference to food. The Journal aims to be informative to all who generate or make use of toxicological data.

Instructions to Authors

General. Original papers, reviews and letters to the editor may be submitted for publication. Manuscripts from the USA and Canada should be sent to the American Editor, Dr L. Golberg. All others must be sent to the Assistant Editor or to the appropriate Regional Editor. Letters 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 Chemical Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

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Frequency. The Journal will be published bi-monthly.

Printed in Great Britain by A. Wheaton & Co. Ltd, Exeter