Volume 17 Number 5 October 1979

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Food and Cosmetics Toxicology

An International Journal published for the British Industrial Biological Research Association

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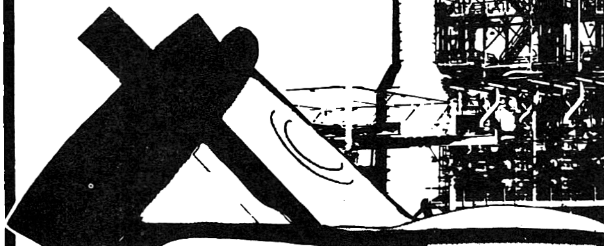

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The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The Journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

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Edited by **Elvira Di Ferrante**, *Commission of the European Communities (CEC), Brussels, Belgium*

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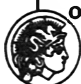
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Research Section

UPTAKE, DISTRIBUTION AND METABOLISM OF [^{14}C]AMARANTH IN THE FEMALE RAT

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Abstract—Four female rats were dosed by gavage with [^{14}C]amaranth (20 mg/kg). Total radioactivity was measured in blood samples taken at specific times over a 24-hr period. The change in blood radioactivity with time after dosing was fitted to a one-compartment mathematical model. The mean times taken to reach maximum concentration of radioactivity and for the decline to 50% of the maximum concentration were 4·2 and 12·5 hr, respectively. Subsequently, two groups of four female rats were dosed with [^{14}C]amaranth (as above) and killed 4 and 12 hr after dosing. The distribution of ^{14}C-activity in various tissues was measured. Calculations of the mean ^{14}C-activity indicated that 14·2% of the administered dose was retained by the rats in the 24-hr period. No radioactivity was detected in respiratory gases. Radioactivity was detected in stomach, large and small intestine, blood, bile, heart, kidney, liver and lung 4 and 12-hr after treatment, but after 24 hr ^{14}C-activity was no longer detectable in the blood, bile or heart. Over the 24 hr following dosing, 65–86% of the given ^{14}C-activity was recovered in the faeces. Total recoveries of ^{14}C-activity ranged from 80–94% of the dose. Radioactive amaranth, naphthionic acid and as many as five unidentified products were detected in the gastro-intestinal tract contents, urine and faeces by thin-layer chromatography.

INTRODUCTION

During the past decade, considerable controversy and concern has arisen regarding the potential toxicity of amaranth (FD & C Red No. 2). Gonadotoxic and embryotoxic effects (Shtenberg & Gavrilenko, 1970) and the reported carcinogenicity of amaranth (Andrianova, 1970; Baigusheva, 1968) provided the impetus for much of this concern.

Subsequent reproduction studies with amaranth in the rat (Collins, Keeler, Black & Ruggles, 1975; Collins & McLaughlin, 1972, 1973; Collins, Ruggles, Holson, Schumacher, Gaylor & Kennedy, 1976; Khera, Przybylski & McKinley, 1974), mouse (Larsson, 1975), rabbit (Keplinger, Wright, Plank & Calandra, 1974) and cat (Khera, Roberts, Trivett, Terry & Whalen, 1976) did not demonstrate any untoward effects on reproduction or on the development of the young. Amaranth gave a negative result in the *B. subtilis* recombination-assay (Kada, Tutikawa & Sadaie, 1972) and in the Ames test (Stoltz, Bendall, Stavric & Munro, 1978). A dominant lethal study using mice was negative (Arnold, Kennedy, Keplinger & Calandra, 1976). Mannell, Grice, Lu & Allmark (1958) and Rubenchik & Bykorez (1963) did not attribute any tumorigenic properties to amaranth but a controversial cancer study (Boffey, 1976) led to the withdrawal of its provisional listing in the USA (*Federal Register*, 1976, **41**, 5823).

The reasons for the controversial results arising from the various amaranth studies are important both from the scientific and from the regulatory viewpoint. It has been suggested that the studies, where untoward toxicological effects were observed (Andrianova, 1970; Baigusheva, 1968), may have resulted from using amaranth of unknown purity (Stavric, Stoltz & Klassen, 1978). Differences in diet (Ershoff & Thurston, 1974) and in the strain and species of the test animal have also been suggested. A consideration of these factors prompted the present study on the uptake, distribution, elimination and metabolism using [^{14}C]amaranth.

EXPERIMENTAL

Animals, treatment and tissue sampling. Four female Wistar rats (200 g, Woodlyn Farms, Guelph, Ontario) were surgically prepared with indwelling jugular vein cannulae (Van Petten, Evans & Salem, 1970). After 12 hr without food, each rat was orally dosed with [1,4,5,8,1′,4′,5′,8′-^{14}C]amaranth [FD & C Red No. 2′ C.I. 16185; C.I. Acid Red 27; 2,7-naphthalenedisulphonic acid 3-hydroxy-4-[4-sulpho-1-naphthalenyl)-azotrisodium salt] (20 mg/kg body weight) in distilled water. The crystalline amaranth (Amersham/Searle, Chicago, IL, USA) was found to be 99% pure by thin-layer chromatography and had a specific activity of 26·7 μCi/mg.

Coprophageal cups were fitted on the rats immediately after dosing and the animals were housed individually in glass metabolism cages equipped with a CO_2 trapping apparatus for the exhaust air. The trapping solvent was ethanolamine–cellosolve (1:1, v/v). Rats had free access to food and water after dosing. Triplicate 25-μl blood samples were taken from the jugular vein cannula every hour for the first 12 hr and at 16

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and 24 hr after dosing. Faeces and urines were collected at intervals of 0–6, 6–12 and 12–24 hr, and kept frozen (−4°C) until they were analysed for radioactivity.

Fifteen minutes before the 24-hr blood sample, the rats were anaesthetized and a cannula was inserted into the bile-duct in order to obtain three 25-μl bile samples from each rat. The animals were then exsanguinated via the abdominal aorta and autopsied. Kidneys, liver, brain, heart, lung and the complete gastro-intestinal (GI) tract were removed. The GI tract was divided into the oesophagus, stomach, small intestine (pyloric sphincter to caecum) and large intestine (caecum to anus). Before removing the urinary bladder, the ureter was tied and the bladder rinsed with 1 ml of water. Contents in the gut were removed and frozen and gut tissues, which had been thoroughly rinsed in 10 ml of distilled water, were blotted dry and weighed. The rinses from the bladder and GI tract were saved for analysis of radioactivity. All the tissues were weighed after the autopsy.

Two further groups of four rats were subsequently dosed with [^{14}C]amaranth and autopsied at 4 and 12 hr after treatment, respectively, following the procedures described above.

Determination of radioactivity. Triplicate samples of approximately 100 mg of all tissues, except kidneys, were solubilized in glass scintillation vials with 1·0 ml of Soluene (New England Nuclear, Boston, MA, USA). Twenty-five μl of urine, blood and the washings of the gut and urinary bladder were similarly solubilized. Both kidneys from each rat were completely digested with Soluene (1 ml/100 mg) and then three 1·0-ml samples were transferred to scintillation vials. Spectrofluor (15 ml; Amersham/Searle) was added to all vials.

Faeces and GI contents which had been stored in glass beakers were removed from the freezer and enough ethanol–water (4:1, v/v) was added to cover the sample. These samples were allowed to swell over the next 3 days and were periodically macerated at room temperature with a glass rod. It was ensured that, at all times, the samples were covered with the ethanol–water mixture. Since a separation of the original metabolic products by thin-layer chromatography was planned, ethanol was added to arrest gut flora metabolism. About 15-mg triplicate samples of the slurry of faecal or GI contents were weighed accurately in plastic scintillation vials. To each vial were added 0·4 ml 30% hydrogen peroxide and 0·2 ml 70% perchloric acid and the samples were digested overnight at room temperature with shaking (Mahin & Lofberg, 1970). 2,5,-Diphenyloxazole (8 ml) in toluene (6 g/litre) and 7 ml cellosolve were then added prior to the determination of radioactivity.

Levels of ^{14}C-activity in the samples were determined with a Beckman LS 230 scintillation counter. Samples were counted for 20 min each and the data were recorded on paper punch-tape which was processed in a Nova 840 mini-computer using a program designed to express scintillation data as dpm/g wet tissue. Counts for a sample were considered significant if they were more than two and a half times the background count. An external standard was used to correct for quenching.

Kinetics. After plotting the blood concentration data on semilogarithmic paper, as dpm/ml against time, it appeared that the uptake and elimination kinetics for the [^{14}C]amaranth in the blood 4 hr after dosing could be described in terms of two first-order processes and a one compartment open-ended model. The radioactive concentration in the blood at any time (t) would then be given by:

$$y(t) = A(e^{-Bt} - e^{-Ct}) \quad \ldots(1)$$

where A is the common pre-exponential parameter, and B and C are the rate coefficients for uptake and elimination, respectively. Estimates of the rate coefficients were obtained from the semilogarithmic plot by the method of curve stripping (Mayersohn & Gibaldi, 1971). These estimates and the original data were used together with a computer program (BMDP non-linear regression; Dixon, 1975) to obtain more precise estimates of the parameters.

The time at which blood concentration reached a maximum and the time at which the concentration had declined to 50% of the maximum ^{14}C-activity was estimated for each animal using equation 1. Analysis of variance was performed on the maximum ^{14}C-activity attained, on the time taken to reach maximum ^{14}C-activity and the time taken for activity to decline to 50% of the maximum ^{14}C-activity. On this basis the two additional groups of rats were autopsied at 4 and 12 hr after treatment and these groups were used to examine further the distribution of radioactivity at these times.

Determination of metabolites and binding. A qualitative analysis of metabolites using TLC was carried out on the samples that were taken at 4 hr from the GI tract contents and 24-hr faeces and urine samples. Samples of GI tract contents and faeces were repeatedly washed with distilled water, centrifuged (10,000 **g**) and the radioactivity measured in the supernatants until no radioactivity could be detected. A comparison of the total radioactivity in supernatants with that in the total sample provided an estimate of the unbound radioactivity.

Urine samples were mixed with twice their volume of absolute ethanol. The precipitated solids were removed by centrifuging and analysed for ^{14}C-activity. In all cases this residue was found to have a negligible ^{14}C-activity. The supernatant was lyophilized, 0·5 ml of water was added to the lyophilized sample and the ^{14}C-activity in a 50-μl sample was determined.

TLC plates (LQD, Kontes/Quantum TLC Systems, Fairfield, NJ, USA) were spotted with the samples from the gut contents, faeces or urine. A [^{14}C]amaranth standard that contained approximately the same amount of ^{14}C-activity and a non-radioactive sample of naphthionic acid (NA) were similarly spotted onto the same plate. The TLC plates were developed in *n*-propanol:ethyl acetate:water (6:1:3 by vol.) and subsequently examined under UV light to determine the location of NA by its blue fluorescence. The plates were then exposed to X-ray film (Kodak XR-1, Toronto, Ontario) for 1–2 wk and the film was then developed to detect the location of ^{14}C-activity.

RESULTS

All four rats showed a rapid uptake of ^{14}C-radioactivity within the first hour after dosing (Fig. 1). Rat 1 showed a decline in ^{14}C-activity in the blood

for 3 hr after dosing followed by an increase until 6 hr after dosing before a progressive decline to 16 hr after dosing (Fig. 1a). Rats 2 and 4 showed a plateau in blood ^{14}C-activity until 5 to 6 hr after dosing which was followed by a decline to near undetectable levels by 16 hr after dosing (Fig. 1b, d). Rat 3 showed some oscillations in the levels of blood ^{14}C-activity for the first 2 to 3 hr after dosing, then a slow decline until 9 hr followed by a more rapid decline until 16 hr after dosing (Fig. 1c). By 24 hr after dosing, blood ^{14}C-activity was below detection in all four rats. The average level of total ^{14}C-activity in the blood at all sampling times for the four rats remained relatively constant from 1 to 6 hr after dosing, then declined exponentially until 16 hr after dosing (Fig. 1e).

It was apparent that the uptake of radioactive components from the gut varied substantially (Fig. 1), probably as a consequence of successive uptake as the dose progressed down the GI tract. However, if the first 2 hr of data were disregarded, the remaining data (i.e. 3 to 16 hr for all rats) followed a first-order exponential elimination which was predicted by the one compartment model. The estimated coefficients for the model (equation 1) derived from the data collected from 3 to 16 hr together with the time to maximum and time to 50% of maximum radioactivity in the blood are shown in Table 1. The mean rate coefficient for the elimination phase was 0·437/hr ($t_{1/2}$ = 5·05 hr). Extrapolation of the terminal phase of elimination of radioactivity from the blood to the lower detection limit for the radioactivity (2800 dpm/ml of blood or 80 dpm/0·025 ml of blood), indicated

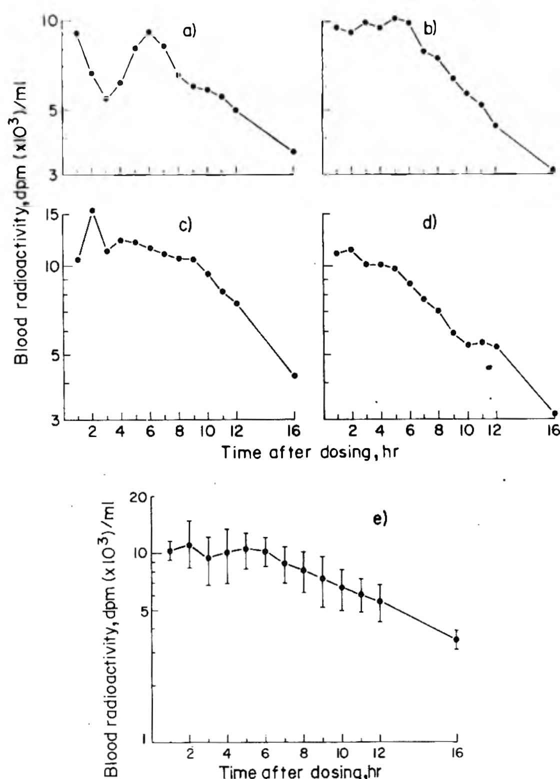

Fig. 1. Radioactivity (dpm/ml) in blood for each female rat (a) no. 1, (b) no. 2, (c) no. 3, (d) no. 4 and (e) combined data for the four rats at specified times after a single oral dose of 20 mg/kg [^{14}C]amaranth.

undetectable levels of radioactivity in blood by 17·5 ± 0·6 hr.

No radioactivity was detected in the brain at 4, 12 or 24 hr after dosing with [^{14}C]amaranth. Neither was any activity found in the respiratory gases. The stomach and the large and small intestine contained the highest amount of radioactivity at 4 and 12 hr after dosing followed by the kidney, lung, heart and liver, bile, serum and blood (Table 2). No radioactivity was detected in blood, serum, bile or heart 24 hr after dosing and, at this sampling time, the large intestine was found to have the highest level of radioactivity followed by the kidney, small intestine, stomach, lung and liver.

The percentages of the dose in various samples collected at 4, 12 and 24 hr after dosing are shown in Table 3. The percentage of ^{14}C-activity from the rats ranged from 80 to 92%, 84 to 89% and 80 to 94% of the dose in animals killed at 4, 12 and 24 hr after dosing, respectively (Table 3). Over a 24-hr period 71·9 to 92·0% of the ^{14}C-active dose was eliminated in the faeces and urine (Table 3). The percentages of the dose retained by the rats (total dose minus faeces, urine, GI tract contents and GI tract rinses) after 12 or 24 hr ranged from 6·1 to 19·7% (mean ± SEM = 14·2 ± 3·3%).

Radiochromatograms of the TLC plates of urine and washings of the GI tract contents and faeces are shown in Fig. 2. The R_F values for amaranth and naphthionic acid (NA) were 0·75 and 0·86, respectively. No radioactive NA was detected in the stomach contents 4 hr after dosing (Fig. 2a, S) and a double band was observed, with an R_F (0·74) that was similar to that of amaranth. Radiochromatograms of TLC plates of washings of the small and large intestinal contents revealed bands with R_Fs corresponding to amaranth and NA, and several additional unidentified bands (Fig. 2a, SI and LI). Radiochromatograms of TLC plates of faecal washings indicated the presence of NA and amaranth plus five unidentified bands (Fig. 2b, F). NA was identified in all urine samples (Fig. 2c) while traces of amaranth, identified only by its R_F, were found in urine samples up to 12 hr after dosing.

Analysis of the washings from the 4-hr samples of the small intestine, large intestine and stomach showed that an average of 61·4, 65·1 and 90·7% respectively of the known total content was retained in the bound state. Stomach contents were visibly pink in colour even after repeated washings. Attempts to extract the residual colour with 1 N-HCl or 1 N-NaOH solutions were unsuccessful. Washing of the 0–6, 6–12 and 12–24 hr faeces samples showed that an average of 65·3, 69·7 and 67·7% respectively of the radioactivity was also in the bound state.

DISCUSSION

The initial phase (up to 2 hr) of uptake of radioactivity from the GI tract following oral dosing with [^{14}C]amaranth was quite variable between rats (Fig. 1a–d). Nevertheless, the levels of ^{14}C-activity were almost at a maximum 1 hr after dosing (at the first blood sample) indicating that the uptake of radioactivity occurred rapidly. At 2 hr after dosing the profile of ^{14}C-activity in blood was similar to that

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Table 1. *Kinetic parameters of the radioactivity in rat's blood after oral dosing with an aqueous solution of [^{14}C]amaranth (20 mg/kg)*

Animal	Coefficient* A (dpm/ml)	Coefficient* B (hr^{-1})	Coefficient* C (hr^{-1})	Time taken for radioactivity to reach a maximum† (hr)	Maximum radioactivity level† (dpm/ml)	Time taken for radioactivity to decline to 50% of maximum† (hr)	Time taken for radioactivity to decline below 2800 dpm/ml‡ (hr)
1	184,574	0·1689	0·1889	5·60	7595	15·0	17·6
2	21,942	0·1268	0·4966	3·69	10,232	11·4	16·2
3	46,534	0·1432	0·3346	4·43	14,107	12·5	19·5
4	17,077	0·1075	0·7276	3·08	10,447	11·0	16·8
Mean ± SEM	67,500 ± 35,700	0·137 ± 0·011	0·437 ± 0·094	4·20 ± 0·44	10,600 ± 1700	12·5 ± 0·7	17·5 ± 0·6

*Estimated from equation (1) in text.
†Based on data collected 2–24 hr after dosing.
‡This was assessed as the limit of detection.

Table 2. *Tissue radioactivity levels and tissue:blood ratios of female rats after gavage with 20 mg/kg [^{14}C]amaranth*

Time after dosing (hr)	Rat no.	Blood radioactivity (dpm/g)	Tissue radioactivity levels (dpm/g) and (in brackets) tissue:blood ratios								
			Serum (× 10^2)	Bile (× 10^2)	Heart (× 10^3)	Kidney (× 10^4)	Liver (× 10^3)	Lung (× 10^3)	Stomach (× 10^5)	Small intestine (× 10^5)	Large intestine (× 10^5)
4	5	284·7	5·95 (2·1)	ND	7·4 (25·9)	8·63 (303·1)	8·84 (31·0)	6·43 (22·6)	4·07 (1429·5)	4·46 (1566·6)	8·67 (3045·3)
	6	270·6	2·56 (1·0)	ND	5·7 (21·1)	3·60 (133·0)	7·77 (28·7)	7·26 (26·8)	2·94 (1086·4)	7·97 (2945·3)	1·64 (606·1)
	7	278·7	5·33 (1·0)	23·43 (8·4)	8 6 (30 8)	16·27 (583·8)	7·08 (25·4)	15·45 (55·4)	1·83 (656·6)	2·71 (972·4)	16·82 (6035·2)
	8	673·8	9·41 (1·4)	77·22 (11·5)	6·4 (9·5)	15·24 (226·1)	16·19 (24·0)	12·95 (19·2)	17·66 (2620·9)	12·35 (1832·8)	15·09 (2241·0)
12	9	163·0	ND	ND	0·9 (5·5)	1·24 (76·1)	3·36 (20·6)	3·52 (21·6)	0·21 (128·8)	0·15 (92·0)	7·01 (4300·6)
	10	181·1	2·95 (1·6)	4·30 (2·4)	1·9 (10·5)	7·8 (430·7)	2·94 (16·2)	5·58 (30·8)	0·26 (143·5)	10·18 (5621·2)	1·25 (690·2)
	11	125·9	2·03 (1·6)	3·63 (2·9)	NS	9·83 (780·7)	4·05 (32·2)	3·77 (29·9)	0·10 (79·4)	0·09 (71·4)	11·94 (9483·7)
	12	398·2	5·82 (1·5)	12·78 (3·2)	5·9 (14·8)	18·44 (463·0)	12·99 (32·6)	12·84 (32·2)	0·15 (37·7)	0·28 (70·3)	40·68 (10,215·9)
24	1	NS	NS	NS	NS	0·69	0·76	1·04	0·02	0·02	0·56
	2	NS	NS	NS	NS	1·27	1·22	1·26	0·02	0·02	2·52
	3	NS	NS	NS	NS	0·15	1·55	2·61	0·07	0·02	1·29
	4	NS	NS	NS	NS	0·59	0·08	0·15	NS	0·01	0·12

ND = not determined NS = not significantly above background

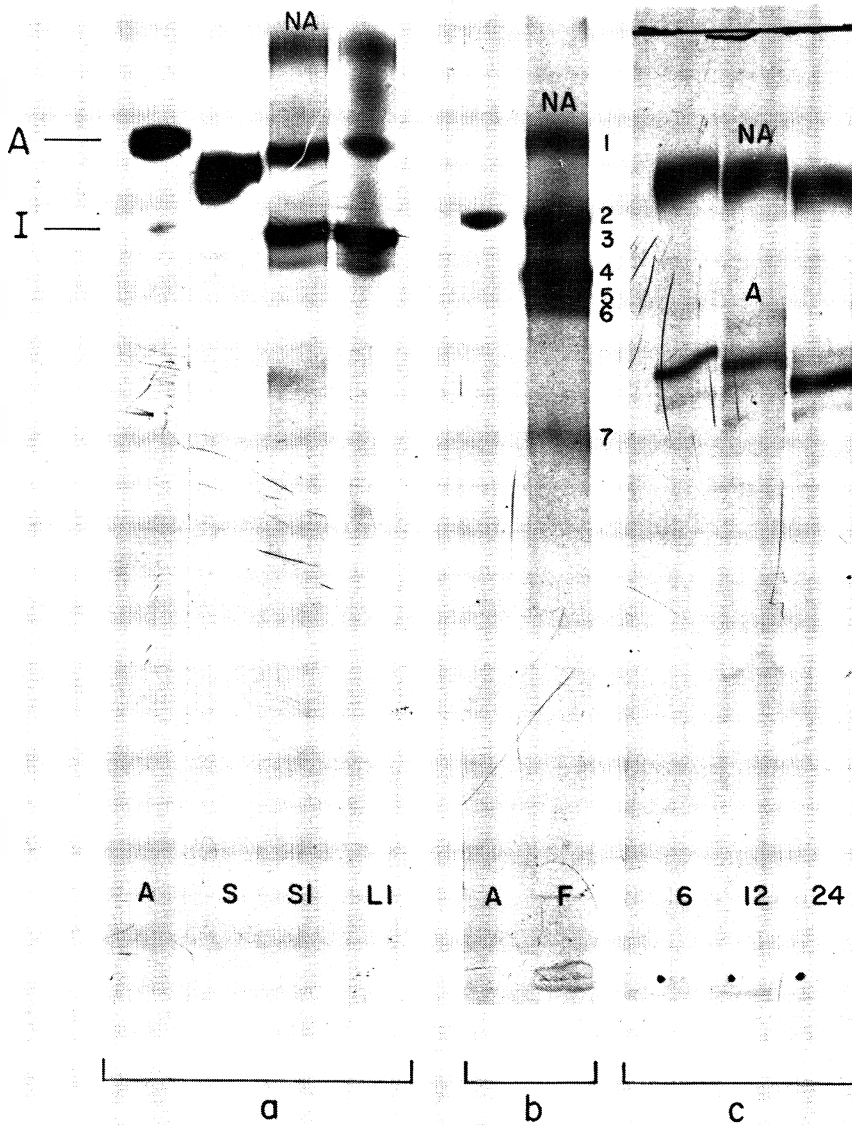

Fig. 2. Radiochromatographs of TLC plates of radioactive products in urine, faeces and different tissues of rats orally dosed with [^{14}C]amaranth: (a) Water-soluble products from GI tract contents of rats killed 4 hr after dosing: A-amaranth (standard); I-unidentified impurity in amaranth standard; S-stomach; SI-small intestine; LI-large intestine; NA-naphthionic acid. (b) Water-soluble products from faeces: A-amaranth (standard), and F-faecal extract. The numbers (1)–(7) are: (1) NA, (2) amaranth and (3)–(7) unknowns. (c) Urine collected for the first 24-hr period after dosing: 6, 12 and 24 represent collection periods 0–6, 6–12, and 12–24 hr, respectively.

Table 3. *Percentage of the radioactive dose in urine, faeces, ingesta and tissues of female rats after dosing with [^{14}C]amaranth (20 mg/kg body weight)*

Source	Time after dosing (hr)....	Percentage of radioactivity dose											
		4				12				24			
	Rat no....	5	6	7	8	9	10	11	12	1	2	3	4
	Dose (dpm × 10^8)....	2·8	3·1	2·6	2·4	2·7	2·4	3·4	2·5	2·6	2·1	2·2	2·6
Urine		0·5	1·0	1·7	0·8	4·2	5·4	5·0	10·7	10·3	7·2	6·6	5·6
Faeces		NS	0·01	NS	0·01	50·1	33·9	45·2	12·4	68·7	75·4	65·3	86·4
Stomach contents		27·8	17·9	0·32	4·9	0·1	5·9	0·01	0·01	0·01	NS	NS	NS
Small-intestinal contents		17·3	12·3	9·4	20·8	0·2	9·2	0·04	0·1	0·02	0·01	0·02	NS
Large-intestinal contents		39·6	44·3	66·0	45·6	27·7	30·8	34·6	52·6	1·2	5·9	8·1	1·9
GI tract rinses		2·5	2·7	4·2	5·9	1·2	1·8	1·0	4·9	0·06	0·03	0·2	0·03
All tissues		4·1	1·5	2·9	5·3	0·8	2·5	2·0	4·1	0·1	0·03	0·1	0·1
Percentage recovery		91·8	79·7	84·5	83·3	84·3	89·5	87·8	84·8	80·4	88·6	80·3	94·0

NS = No radioactivity detected

reported for NA (Pritchard, Holmes & Kirschman, 1976). These authors indicated that serum NA concentrations plateaued from 2 to 8 hr after dosing then followed a first-order elimination phase. In the present studies, the times to peak and to decline to 50% of the peak concentration of ^{14}C-activity in plasma were 4·2 ± 0·4 and 12·5 ± 0·7 hr respectively.

There are two important points regarding the plasma radioactivity uptake/elimination profiles following oral administration of [^{14}C]amaranth. First, the ^{14}C-activity in plasma could be derived from [^{14}C]amaranth *per se* and/or from various metabolic breakdown products of amaranth. The uptake of amaranth *per se* from the GI tract has been reported (Radomski & Mellinger, 1962). This is further supported by our data which indicated the presence of traces of [^{14}C]amaranth in urine, although this amaranth was identified by its R_F value on radiochromatograms only (Fig. 2c). The fact that metabolic breakdown products of amaranth could contribute to the radioactivity in plasma was suggested by the identification (by R_F values and fluorescence) of NA and several other metabolites in radiochromatograms of lyophilized washings of ingesta from the large and small intestines (Fig. 2a) and urine (Fig. 2c). In addition to NA, Singh (1970) has identified 1-amino-2-naphthol-3,6-disulphonic acid (amino-R-acid) and 1,2-naphthoquinone-3,6-disulphonic acid as metabolites of amaranth in urine. Consequently, it is reasonably certain that the ^{14}C-activity in rat plasma represented a mixture of amaranth and amaranth metabolites.

The second point related to the plasma ^{14}C-activity profiles (Fig. 1a–c) is that the observed elimination phase of the radioactivity from plasma was undoubtedly much longer than the simple elimination of NA or other metabolites from plasma as would be observed, for example, following iv dosing. The azoreduction of amaranth by the gut microflora occurs within the small and large intestines (Roxon, Ryan & Wright, 1967; Walker, 1970) as the digesta passes through the GI tract. Consequently, the uptake of ^{14}C-activity from the GI tract would have occurred during the time period required for GI tract transit of digesta. As a result, the apparent elimination phase of the ^{14}C-activity from plasma would be extended due to the continued uptake of radioactivity from the GI tract as amaranth was reduced.

Regardless of these two confounding factors concerning the plasma ^{14}C-activity profiles, the profiles do demonstrate the length of time that amaranth or its various breakdown products are present in blood. The data indicate that the majority of ^{14}C-activity was cleared from the body within 24 hr and that any accumulation of amaranth in the body which may occur during long-term exposure could only result from a minor proportion of the ^{14}C-activity. This is further supported by the fact that the level of ^{14}C-activity decreased in all tissues between 4 and 12 hr after dosing, except for the kidney and GI tract which are major pathways of excretion. However, a complete assessment of the possible accumulation of amaranth or its metabolites requires data from multidose pharmacokinetic studies.

Radomski & Mellinger (1962) reported that rats absorbed 18% of an oral dose of NA while Pritchard *et al.* (1976) calculated 17·2% of absorption of NA from fasted rats and 10·2% from non-fasted rats. These data were similar to the mean retention (14·2%) of ^{14}C-activity observed in the present studies following oral administration of [^{14}C]amaranth to rats. These data indicate that a major proportion of the ^{14}C-activity taken up was probably NA and other amaranth metabolites.

Pritchard *et al.* (1976) demonstrated effects of feeding on serum levels of NA in rats and Ershoff & Thurston (1974) demonstrated a relationship between type of diet and amaranth toxicity. In the present studies, repeated washing of stomach contents with water until no ^{14}C-activity could be detected in the supernatant, revealed that 90·7% of the ^{14}C-activity present in the stomach contents was not water soluble. Further treatment of the stomach contents with 1 N HCl or NaOH did not remove the ^{14}C-activity indicating that the ^{14}C-activity was quite firmly bound to the food components. Similar treatment of the small and large intestinal contents demonstrated that 61·4 and 65·1%, respectively, of the ^{14}C-activity present was not water soluble. Similarly, the percentage of water insoluble ^{14}C-activity in faeces collected from 0 to 6, 6 to 12 and 12 to 24 hr after dosing were 65·3, 69·7 and 67·7%, respectively. Digestive processes acting on the GI tract contents were probably responsible for the increase in water soluble ^{14}C-activity in intestinal contents and faeces relative to the stomach contents. These data support the hypothesis, pro-

posed by Ershoff & Thurston (1974), that binding to various materials in the digesta accounts for the variation in the toxicity of amaranth to animals on different diets.

This interaction of amaranth with various dietary components may have been responsible for the variation in uptake/elimination profiles of ^{14}C-activity observed between rats in the first few hours after dosing with [^{14}C]amaranth (Fig. 1a–d). Differences in food consumption were associated with variations in the uptake and tissue distribution of ^{14}C-activity in rats autopsied 4 hr after dosing. It was observed that rat 7 (Table 2) consumed food immediately after dosing while rat 8 did not. Tissue–blood concentration ratios for the stomach, small intestine and large intestine for rat 7 were 656, 972 and 6035, respectively, whereas rat 8 had ratios of 2620, 1832 and 2241, respectively (Table 2). Radioactivity in the blood of rat 7 was 278·7 dpm/ml while that for rat 8 was 673·8 dpm/ml. The total ^{14}C-activity retained in the tissues was 2·9 and 5·3% of the total dose for rats 7 and 8, respectively. These data indicate that the consumption of food immediately after dosing resulted in generally lower tissue:blood concentration ratios, lower blood ^{14}C-activity levels and a concomitant lower percentage retention of ^{14}C-activity. From these studies, it is evident that the presence of food in the GI tract alters the uptake and/or elimination of amaranth. Further studies are necessary to elucidate the interactions between diet and the pharmacokinetics of amaranth and to determine the importance of these factors in the toxicological assessment of amaranth.

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EFFECTS OF CALCIUM AND SODIUM CARRAGEENANS AND ι-CARRAGEENAN ON HAMSTER FOETAL DEVELOPMENT

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Abstract—The foetal effects of calcium κ,λ-carrageenan, sodium κ,λ-carrageenan and ι-carrageenan (C-16) were studied in Syrian hamsters. Dose levels of 10, 40, 100 or 200 mg/kg were given by oral intubation on days 6–10 of gestation. No dose-related teratogenic or foetotoxic effects occurred with any of the three compounds tested.

INTRODUCTION

Carrageenan is the generic name for the calcium, sodium, potassium and other salts of sulphated polysaccharides extracted from red algae and used extensively as gelling, thickening and emulsifying agents in food, pharmaceuticals and cosmetics. Three main types, kappa (κ), lambda (λ) and iota (ι), can be differentiated on the basis of type, location and number of sulphate groups. Carrageenans used in food are of high molecular weight (over 100,000) and usually are mixtures of κ- and λ-carrageenan. Degraded carrageenan, usually ι-carrageenan, is of low molecular weight and is not permitted in food in the United States, but it is used as an antipeptic agent in some European countries.

Despite the ever-increasing use of the high-molecular-weight compound, relatively few studies have been performed to test its effects on foetuses. Frohberg, Oettel & Zeller (1969) reported a slight decrease in the percentage of live foetuses and the occurrence of one cleft palate in 82 live offspring after ip injection of 1% aqueous carrageenan mucilage (type unspecified) into pregnant mice on days 11–15 of gestation. When high-molecular-weight calcium κ,λ-carrageenan in corn oil suspension was administered to pregnant mice, rats, rabbits and hamsters by intubation, the average number of resorptions increased and foetal weight decreased in mice and rats, but no differences appeared in rabbits and hamsters (Food and Drug Research Laboratories, Inc., 1973a). In a similar study of the same species intubated with high-molecular-weight sodium κ,λ-carrageenan in corn oil suspension, foetal weight decreased in mice and rats and the number of resorptions increased in mice, but hamsters and rabbits showed no compound-related foetal effects (Food and Drug Research Laboratories, Inc., 1973b). Because of the apparent variations between species, the two compounds were retested in the same strains of rats and hamsters. The compounds were fed in the diet at levels of 1·0 or 5·0% to pregnant rats during days 6–15 of gestation and to pregnant hamsters during days 6–10 (Food and Drug Research Laboratories, Inc., unpublished data 1973, in FDA files). Foetal body weights and the numbers of implantation sites, live and dead foetuses, resorption sites and abnormalities showed no dose-related effects.

More recently, the effects of the dietary intake of calcium κ, λ-carrageenan were investigated in a three-generation reproduction and teratology study in rats (Collins, Black & Prew, 1977a,b). No dose related effects were observed in either mothers or offspring except for significant decreases in the weights of offspring at weaning. No studies on the possible foetal effects of ι-carrageenan have been found in the literature.

In all except one of the studies reported above, the carrageenan compound was administered in corn oil or in the diet; the exception was the administration of unspecified aqueous carrageenan mucilage (Frohberg *et al.* 1969). In the study reported here carrageenan was administered in water. This work is part of a series of Food and Drug Administration studies of the foetal effects of carrageenan compounds and other food additives.

EXPERIMENTAL

Materials and animals. Calcium κ,λ-carrageenan (Gelcarin-HMR; Lot no. 282400) and sodium κ,λ-carrageenan (Viscarin; Lot no. 421602) were obtained from Marine Colloids, Inc., Rockland, ME. Iota-carrageenan, C-16 (Poligeenan; Batch L-4006) was obtained from Laboratoires Glaxo, Paris, France. The major components of the compounds were: calcium κ,λ-carrageenan—sulphate ester 24·4%, 3,6-anhydrogalactose 22·2%, water 9·0%; sodium κ,λ-carrageenan—sulphate ester 28·6%, water 8·4%; C-16—sulphate ester 33·05%, water 5%. Pregnant Syrian hamsters (*Mesocricetus auratus*) were obtained from Lakeview Hamstery, Newfield, NJ. The animals weighed 114·9 ± 10·2 g (mean ± SD) on day 2 after mating. Purina Lab Chow (Ralston-Purina Co., Inc., St. Louis, MO) and water were available *ad lib.*

Table 1. *Maternal and reproductive data for hamsters intubated with carrageenans*

Compound	Dose level (mg/kg)	No. of pregnant females examined	Mean maternal body-weight gain* (g)	Autopsy findings (mean/dam)					Resorptions per litter (%)	Pre-implantation loss (%)	Females with resorptions (%)		Mean foetal weights (g)	
				Corpora lutea	Implantations	Resorptions		Viable foetuses			One or more	Two or more	Male	Female
						Early	Late							
Calcium carrageenen	0	26	34·3	13·4	13·0	0·6	0	12·5	4·4	2·9	46·2	11·5	1·8	1·7
	10	25	30·6	13·1	12·2	0·9	0·12	11·2	8·8	6·4	60·0	16·0	1·8	1·7
	40	21	33·7	13·3	12·1	0·8	0	11·3	7·5	8·6	47·6	14·3	1·8	1·8
	100	22	30·8	12·5	11·9	0·9	0·04	11·0	8·0	5·1	22·7	13·6	1·9	1·8
	200	25	35·4	13·5	13·1	0·5	0·04	12·6	4·0	3·2	40·0	8·0	1·9	1·8
Sodium carrageenan	0	21	34·0	13·8	12·7	0·6	0·05	12·0	5·4	8·0	57·1	4·8	1·8	1·8
	10	22	33·7	13·4	13.1	0·6	0	12·5	5·0	2·4	54·6	9·1	1·8	1·8
	40	22	36·0	13·8	12·9	0·8	0·04	12·0	7·6	6·9	63·6	22·7	1·8	1·8
	100	23	38·1	13·1	12·6	0·4	0·09	12·1	3·7	4·0	43·5	4·4	1·8	1·8
	200	24	33·2	13·2	12·5	0·7	0·08	11·8	5·6	5·1	54·2	16·7	1·8	1·8
C-16	0	8	32·6	12·6	12·2	0·8	0	11·5	5·9	3·0	50·0	25·0	1·8	1·8
	10	8	34·2	13·5	13·1	0·4	0	12·8	3·3	2·8	37·5	0	1·8	1·8
	40	8	35·0	13·9	12·6	0	0·12	12·5	1·8	9·0	12·5	0	2·0	1.9
	100	8	35·5	12·4	11·8	0·4	0	11·4	3·0	5·0	25·0	12·5	1·9	1·9
	200	8	36·2	13·1	13·3	0·9	0	12·4	6·8	0	50·0	37·5	1·9	1·8

*Maternal body-weight gain between days 2 and 14 of gestation.

Table 2. *Incidence of specific external and soft-tissue variations in foetuses of hamsters intubated with carrageenans*

Variation	Incidence* in foetuses and litters of dams treated with . . .														
	Calcium carrageenan					Sodium carrageenan					C-16				
Dose level (mg/kg) . . .	0	10	40	100	200	0	10	40	100	200	0	10	40	100	200
External examination															
No. examined	324 (26)	280 (25)	238 (21)	242 (22)	314 (25)	253 (21)	275 (22)	264 (22)	279 (23)	282 (24)	92 (8)	102 (8)	100 (8)	91 (8)	99 (8)
Haemorrhage	1 (1)			1 (1)	2 (2)		1 (1)	2 (2)	1 (1)	2 (2)					
Exencephaly		1 (1)	1 (1)							1 (1)					
Fused ribs		1 (1)	1 (1)										1 (1)		
Haematoma							2 (2)	1 (1)							
Swollen left shoulder											1 (1)				
Soft-tissue examination															
No. examined	178 (26)	142 (25)	123 (21)	135 (22)	184 (25)	135 (20)	162 (22)	152 (21)	149 (23)	147 (24)	49 (8)	59 (8)	56 (7)	44 (8)	55 (8)
Haemorrhage, internal	5 (5)	3 (3)	1 (1)	2 (2)	10 (9)			1 (1)	1 (1)	1 (1)			1 (1)		
Oedema	1 (1)						1 (1)								
Hydrocephalus					1 (1)						2 (1)				
Cerebral lobe malformed	1 (1)														
Kidney malformed						2 (2)	4 (4)	1 (1)		2 (2)					
Renal pelvis enlarged	2 (2)	1 (1)	2 (2)	3 (2)	8 (7)		1 (1)	1 (1)							
Ectopic kidney		2 (2)		1 (1)	1 (1)		4 (3)	2 (2)	1 (1)						
Hydroureter						1 (1)	1 (1)								
Mottled liver				1 (1)											
Testes missing	1 (1)														
Testes incompletely descended					1 (1)										

*No. of foetuses affected and, in parentheses, no. of litters.

Experimental procedure. Randomly selected animals were intubated with sodium or calcium carrageenan or C-16 in distilled water at dose levels of 10, 40, 100 or 200 mg/kg, on days 6–10 of gestation. Animals of the control groups were intubated with distilled water on the same days. Day 0 was considered the day on which sperm were found in the vagina. At least 21 pregnant females were examined at each dose level of calcium and sodium carrageenan. Only eight pregnant females were tested at each dose level of C-16 as only a limited supply of this compound was available. The highest level of 200 mg/kg was chosen because the gelling capacity of the compounds precluded higher concentrations.

On day 14, one day before expected parturition, the animals were killed by chloroform and the uteri were examined *in situ* for live and dead and/or resorbed foetuses. Deciduomas, brownish implantation sites without placentas, were called early deaths. Implantation sites with placentas, and with complete but non-viable foetuses that were of subnormal size, showed retarded development or were in a macerated condition were classed as late deaths according to the terminology of Bateman & Epstein (1971) and the MARTA Committee on Terminology (1969). Each live foetus was weighed, sexed and examined for gross external malformations under a dissecting microscope. Corpora lutea were counted under magnification. Approximately half of the foetuses from each litter were fixed in alcohol, stained with Alizarin Red S (Dawson, 1926) and examined for skeletal anomalies. The remaining foetuses were fixed in Bouin's solution, razor-blade sectioned (Wilson, 1965) and examined for internal malformations of the soft tissues.

Statistical analysis. Data on the numbers of corpora lutea per dam and implantations per dam were analysed by a two-tailed t test. The number of resorptions per litter were transformed by use of the Freeman–Tukey arc-sine transformation for binomial proportions (Mosteller & Youtz, 1961) followed by a two-tailed t test. Preimplantation loss data were transformed using the Freeman–Tukey arc-sine transformation followed by an analysis of variance. The number of viable implants and foetal body weights were submitted to an analysis of variance followed by a least significant difference (LSD) test. The numbers of litters with one or more resorptions, one or more abnormalities and specific external, soft tissue and skeletal variations were analysed by the Fischer exact test (Siegel, 1956). Analysis of variance was used to test maternal weight gain. The data on foetuses with one or more abnormalities per litter were transformed by use of the Freeman–Tukey square root transformation for Poisson distribution (Mosteller & Youtz, 1961) followed by a t test.

RESULTS

No adverse external effects of the test compounds were observed in the pregnant hamsters. Maternal reproductive data and foetal weights (Table 1) showed no dose-related effects on maternal body-weight gain between days 2 and 14 or on the numbers of corpora lutea, implantations, early and late resorptions and viable foetuses per litter for any of the compounds tested. Sporadic increases were seen in the percentages of resorptions per litter in groups given calcium and sodium carrageenan but these increases were not dose-related. The percentages of preimplantation loss in the control groups varied (2·9, 8·0 and 3·0%). The increases in preimplantation loss that occurred at the intermediate dose levels of calcium carrageenan and C-16 did not appear to be significant. The numbers of females with one or more and two or more resorptions showed no dose-related effect of any compound. Neither male nor female foetal weights were affected by the compounds.

None of the carrageenans tested produced any compound-related external abnormalities (Table 2).

Table 3. *Incidence of soft-tissue variations in foetuses of hamsters intubated with carrageenans*

Compound	Dose level (mg/kg)	Soft-tissue variations		Foetuses with one or more soft-tissue variations			Litters with one or more abnormal foetuses	
		Total	Mean/ litter	No.	Mean/ litter	% of total foetuses	No.	%
Calcium carrageenan	0	10	0·4	10	0·4	5·6	8	30·8
	10	6	0·2	6	0·2	4·2	5	20·0
	40	3	0·1	3	0·1	2·4	2	9·5
	100	7	0·3	7	0·3	5·2	5	22·7
	200	21	0·8	20	0·8	10·9	14	56·0
Sodium carrageenan	0	3	0·2	3	0·2	2·2	2	10·0
	10	11	0·5	7	0·3	4·3	6	27·3
	40	5	0·2	5	0·2	3·3	4	19·0
	100	2	0·1	2	0·1	1·3	2	8·7
	200	3	0·1	3	0·1	2·0	3	12·5
C-16	0	2	0·2	2	0·2	4·1	1	12·5
	10	0	0	0	0	0	0	0
	40	1	0·1	1	0·1	1·8	1	14·3
	100	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0

Table 4. *Incidence of specific sternebral and skeletal variations in foetuses of hamsters intubated with carrageenans*

Variation	Dose level (mg/kg)...	Incidence* in foetuses and litters of dams treated with ...														
		Calcium carrageenan					Sodium carrageenan					C-16				
		0	10	40	100	200	0	10	40	100	200	0	10	40	100	200
No. examined		144 (26)	132 (25)	113 (21)	98 (18)	128 (25)	117 (21)	111 (22)	104 (21)	121 (23)	129 (24)	42 (8)	41 (8)	44 (8)	47 (8)	42 (8)
Sternebrae:	incomplete	30 (17)	26 (18)	17 (11)	11 (8)	30 (18)	27 (16)	22 (17)	18 (12)	23 (14)	33 (22)	12 (8)	6 (5)	12 (6)	8 (5)	6 (5)
	bipartite	44 (19)	43 (19)	15 (9)	24 (14)	35 (18)	41 (16)	26 (15)	32 (13)	33 (19)	52 (20)	21 (7)	10 (4)	20 (8)	7 (4)	8 (6)
	malaligned	9 (7)	14 (10)	3 (3)	9 (8)	13 (10)	15 (13)	12 (9)	13 (9)	8 (6)	15 (12)	3 (3)	5 (4)	3 (3)	10 (4)	2 (2)
	missing	133 (26)	125 (25)	102 (21)	87 (18)	111 (25)	105 (21)	102 (22)	89 (21)	104 (23)	108 (24)	34 (8)	36 (8)	37 (8)	41 (8)	33 (8)
	fused								1 (1)							
Poorly ossified cranial bones		1 (1)														
Hyoid bone†		10 (7)	1 (1)		2 (1)	1 (1)	1 (1)							1 (1)		
Vertebral centrum‡		1 (1)	1 (1)	1 (1)												
Ribs:	fused		2 (2)		1 (1)						1 (1)					
	wavy			1 (1)												
Thirteenth rib bud								1 (1)	1 (1)							
Supernumerary ribs		32 (17)	40 (18)	44 (19)	25 (13)	33 (17)	32 (16)	30 (13)	21 (11)	36 (17)	43 (15)	10 (5)	14 (7)	8 (5)	20 (7)	14 (5)
Poorly ossified pubis		1 (1)														
Metacarpals†		5 (4)												1 (1)		
Metatarsals†		38 (16)	24 (10)	3 (3)	5 (5)	2 (2)	6 (6)	10 (5)	3 (3)	5 (4)	1 (1)	2 (2)	6 (4)	2 (2)		1 (1)

*No. of foetuses affected and, in parentheses, no. of litters.
†Poorly ossified and missing combined.
‡Poorly ossified and bipartite combined.

Table 5. *Incidence of sternebral variations in foetuses of hamsters intubated with carrageenans*

Compound	Dose level (mg/kg)	Sternebral variations		Foetuses with one or more sternebral variations			Litters with one or more abnormal foetuses	
		Total	Mean/litter	No.	Mean/litter	% of total foetuses	No.	%
Calcium carrageenan	0	216	8·3	144	5·5	100	26	100
	10	208	8·3	129	5·2	97·7	25	100
	40	137	6·5	112	5·3	99·1	21	100
	100	131	7·3	95	5·3	96·9	18	100
	200	189	7·6	126	5·0	98·4	25	100
Sodium carrageenan	0	188	9·0	116	5·5	99·1	21	100
	10	162	7·4	111	5·0	100	22	100
	40	153	7·3	101	4·8	97·1	21	100
	100	168	7·3	119	5·2	98·3	23	100
	200	208	8·7	128	5·3	99·2	24	100
C-16	0	70	8·8	39	4·9	92·9	8	100
	10	57	7·1	38	4·8	92·7	8	100
	40	72	9·0	42	5·2	95·5	8	100
	100	66	8·2	45	5·6	95·7	8	100
	200	49	6·1	38	4·8	90·5	8	100

The numbers of internal haemorrhages and enlarged renal pelvises were increased at the highest dose level of calcium carrageenan but the increases were not statistically significant on a litter basis and the haemorrhages were not concentrated in any single area of the body. Neither sodium carrageenan nor C-16 produced any dose-related soft-tissue abnormalities. There was no significant, dose-related effect on total soft-tissue abnormalities (Table 3).

No compound-related effect on specific skeletal variations occurred (Table 4), nor was there any dose-related effect when data on the sternebral variations (exclusive of the other skeletal variations) were analysed per litter and per total number of foetuses (Table 5). Nearly all of the litters contained one or more foetuses with sternebral variations; the most frequent sternebral defect was a missing fifth sternebra. No differences between control and experimental groups were found when the data were analysed with and without inclusion of the data on the missing fifth sternebrae. The mean skeletal variations (other than the sternebral defects) showed no effect of any compound (Table 6).

DISCUSSION

The lack of evidence of any effects of calcium carrageenan intubation in hamsters in this study correlated with the results of a multigeneration study in which rats were given the compound in the diet (Collins *et*

Table 6. *Incidence of skeletal variations, other than sternebral variations, in foetuses of hamsters intubated with carrageenans*

Compound	Dose level (mg/kg)	Skeletal variations		Foetuses with one or more skeletal variations			Litters with one or more abnormal foetuses	
		Total	Mean/litter	No.	Mean/litter	% of total foetuses	No.	%
Calcium carrageenan	0	88	3·4	62	2·4	43·1	23	88·5
	10	68	2·7	59	2·4	44·7	23	92·0
	40	49	2·3	47	2·2	41·6	20	95.2
	100	33	1·8	30	1·7	30·6	13	72·2
	200	36	1·4	35	1·4	27·3	19	76·0
Sodium carrageenan	0	39	1·9	39	1·9	33·3	18	85·7
	10	41	1·9	38	1·7	34·2	15	68·2
	40	25	1·2	25	1·2	24·0	14	66·7
	100	41	1·8	41	1·8	33·9	19	82·6
	200	45	1·9	45	1·9	34·9	16	66·7
C-16	0	12	1·5	11	1·4	26·2	5	62·5
	10	20	2·5	19	2·4	46·3	7	87·5
	40	12	1·5	9	1·1	20·5	6	75·0
	100	20	2·5	20	2·5	42·6	7	87·5
	200	15	1·9	14	1·8	33·3	5	62·5

al. 1977a,b) and with results obtained by the Food and Drug Research Laboratories, Inc. (1973a) after animals were treated by intubation. In a dietary study with hamsters fed diets containing calcium carrageenan at 1 and 5% levels, the number of animals with evidence of implantations was decreased in animals given the higher level (68% *v.* 82% in the controls) (Food and Drug Research Laboratories, Inc., unpublished data 1973, in FDA files). Although the decrease was not statistically significant, it was speculated that carrageenan, administered at about the time of implantation in the hamster, might affect this process. However no such effect was evident in this present study after hamsters were intubated on days 6–10 of gestation with calcium carrageenan at dose levels of up to 200 mg/kg. The only increases in abnormalities that occurred were in the incidence of internal haemorrhages and of renal pelvis enlargement in foetuses of hamsters given the high level of calcium carrageenan. However, these increases were not statistically significant.

No compound-related foetal effects appeared after sodium carrageenan intubation. These results are similar to those of the intubation study in hamsters (Food and Drug Research Laboratories, Inc., 1973b) and of the dietary study in hamsters and rats (Food and Drug Research Laboratories, Inc., unpublished data 1973, in FDA files).

C-16 seemed to have no effects on foetuses in the eight litters studied. However, additional testing is necessary to confirm these results.

In conclusion, calcium carrageenan, sodium carrageenan and C-16, at any of the levels tested, do not appear to cause teratogenic or foetotoxic effects in hamsters.

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2,6-DI-*TERT*-BUTYL-4-METHYLENE-2,5-CYCLOHEXADIENONE: A HEPATIC METABOLITE OF BUTYLATED HYDROXYTOLUENE IN RATS

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Abstract—Two metabolites of butylated hydroxytoluene were isolated from the livers of orally treated male rats. The major metabolite was identified as 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone, by gas chromatography and mass spectroscopy and by reduction with lithium aluminium hydride. Only non-conjugated unchanged BHT was identified in samples of lung, kidney, pancreas, brain and adipose tissue from these rats.

INTRODUCTION

Butylated hydroxytoluene (BHT), a hindered phenolic antioxidant, can cause haemorrhagic death and a dose-dependent decrease in the prothrombin index in male rats when administered in the diet (Takahashi & Hiraga, 1978a,b). Vitamin K deficiency produced by a direct effect of BHT and/or its metabolites in the liver may be a central factor in this bleeding (Takahashi & Hiraga, 1979). The nature of this effect, which differs from that of warfarin, has not yet been determined, but may be attributable to the structural characteristics of BHT rather than to its antioxidant properties (Takahashi & Hiraga, 1978c). Because of the species, strain and sex differences in the effects of BHT, it seems possible that the haemorrhagic effect is related to BHT metabolism (Takahashi & Hiraga, 1978d).

The major metabolic pathway of BHT *in vivo* involves the oxidation of the 4-methyl group (Hathway, 1966) to yield 2,6-di-*tert*-butyl-4-hydroxymethylphenol (BHT alcohol), 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (BHT aldehyde) and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT acid). These metabolites, however, do not cause haemorrhagic death when administered in the diet (Takahashi & Hiraga, 1978c) and therefore the compound(s) affecting vitamin K metabolism must be either BHT itself or metabolites preceding BHT alcohol. We have now attempted to isolate and identify BHT metabolites in the liver, the site of synthesis of the vitamin K-dependent coagulation factor.

EXPERIMENTAL

Materials. BHT was purchased from Tokyo Kasei Kogyo Co. 2,6-Di-*tert*-butyl-4-methylene-2,5-cyclohexadienone was synthesized by oxidation of BHT with lead dioxide (Filar & Winstein, 1960); the oxidation products included an impurity with the same retention time on Silicone DC-200 as the main quinone methide product and a molecular ion of m/e 220. This compound was apparently 2,6-di-*tert*-butyl-1,4-benzoquinone (Heiss, Zeller & Rieker, 1969). The ultraviolet spectrum (λ_{max} 284 and log ϵ 4·56 in hexane) of 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone, eluted with hexane from the silica-gel column, confirmed the quinone methide structure (Filar & Winstein, 1960).

Animals and treatment. Male Sprague-Dawley rats (body weight *c.* 300 g) were fed a diet containing 1·2% BHT for 2 wk as previously described (Takahashi & Hiraga, 1978a). Some rats died from haemorrhage during the experimental feeding period. At the end of wk 2, the survivors were killed by a blow on the head, and the lungs, kidneys, liver, pancreas and brain and samples of epididymal adipose tissue were dissected out for analysis.

Tissue analyses. Each tissue was mixed with anhydrous sodium sulphate and homogenized with acetone (1 g tissue in 10 ml acetone) using a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland). The acetone solution was evaporated under nitrogen, the residue was dissolved in hexane and the solvent was again evaporated under nitrogen. This residue was dissolved in carbon disulphide for analysis by gas chromatography. Acetone was used for the initial extraction of the tissues because Kitamura, Sumino, Hayakawa & Yano (1977) reported that both BHT and BHT acid could be extracted from acidified urine with acetone but that, with liver tissue, no second peak and no change in the height of the main peak resulted from either acidification or extraction with chloroform–methanol (2:1, v/v).

Gas chromatography was performed on a Shimadzu GC-4BM gas chromatograph equipped with a flame-ionization detector (supplied by Shimadzu Inc., Tokyo). A glass column (3 m × 3 mm id) was packed with Gas Chrom Q containing 10% Silicone DC-200, while a glass cartridge packed with 3·5 cm silanized glass wool and inserted into the port chamber served as a precolumn (Hartman & Rose, 1970). The operating temperatures were 180°C (column), 250°C (detector) and 250°C (injection port) and the carrier-gas (nitrogen) flow rate was 50 ml/min.

Mass spectra were recorded, in some cases after reduction of the compound with lithium aluminium hydride, on a Shimadzu LKB 9000 or JEOL JMS D-300 gas chromatograph–mass spectrometer at 70 eV.

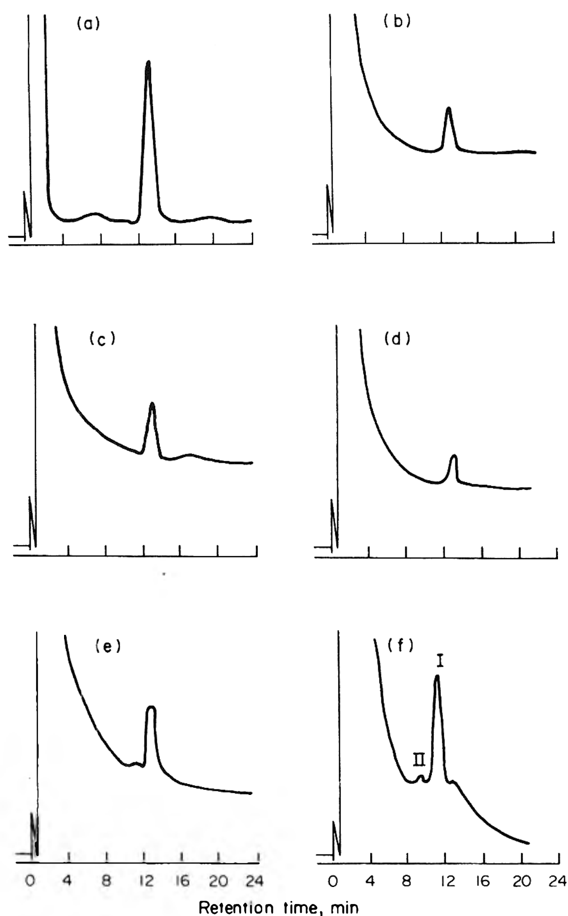

Fig. 1. Gas chromatograms of extracts of (a) adipose tissue, (b) lung, (c) kidney, (d) pancreas, (e) brain and (f) liver from male rats given BHT in the diet. The retention time of BHT was 13 min under the conditions used for each analysis.

RESULTS AND DISCUSSION

Non-conjugated BHT was found in the epididymal adipose tissue, lungs, kidney, pancreas and brain, but not in the liver, in which the presence of a BHT metabolite was demonstrated by gas chromatography (Fig. 1). The mass spectrum of this hepatic metabolite (Fig. 2) showed a molecular ion at *m/e* 218 and the main fragments at *m/e* 203 ($M^+ - CH_3$), *m/e* 175 ($M^+ - CH_3 - C_2H_4$) and *m/e* 161 ($M^+ - C_4H_9$). The same major fragments (apart from the molecular ion) had been shown by Krokhin, Chizhov, Ershov & Volod'kin (1977) to be characteristic of the spectra of several quinone methides, namely α-methyl-, α-deuteromethyl-, α,α-dimethyl-, α-phenyl- and α,α-diphenyl-2,6-di-*tert*-butyl-1,4-methylenequinone. The mass spectrum of the hepatic metabolite corresponded to that of the authentic sample of 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone (Fig. 3) and the gas-chromatographic retention times of the two compounds were the same.

Quinone methides are reduced to the parent phenols by lithium aluminium hydride (Filar & Winstein, 1960), and both the hepatic metabolite and the authentic 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone were converted by this reducing agent at room temperature to BHT, which was identified by gas chromatography–mass spectrometry.

These results identified the main non-conjugated metabolite in the liver as 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone (Fig. 4). The non-conjugated phenols, BHT, BHT alcohol, BHT aldehyde and BHT acid were not clearly demonstrated in the liver although rats given BHT excreted the high-molecular-weight non-conjugated phenols, especially BHT acid, in the urine (Daniel, Gage & Jones, 1968; Takahashi & Hiraga, 1977). Wiebe, Mercer & Ryan (1978) reported finding trace amounts of unchanged BHT but not metabolites in the serum of a man given BHT. It has been reported that 4-hydroxy-4-methyl-

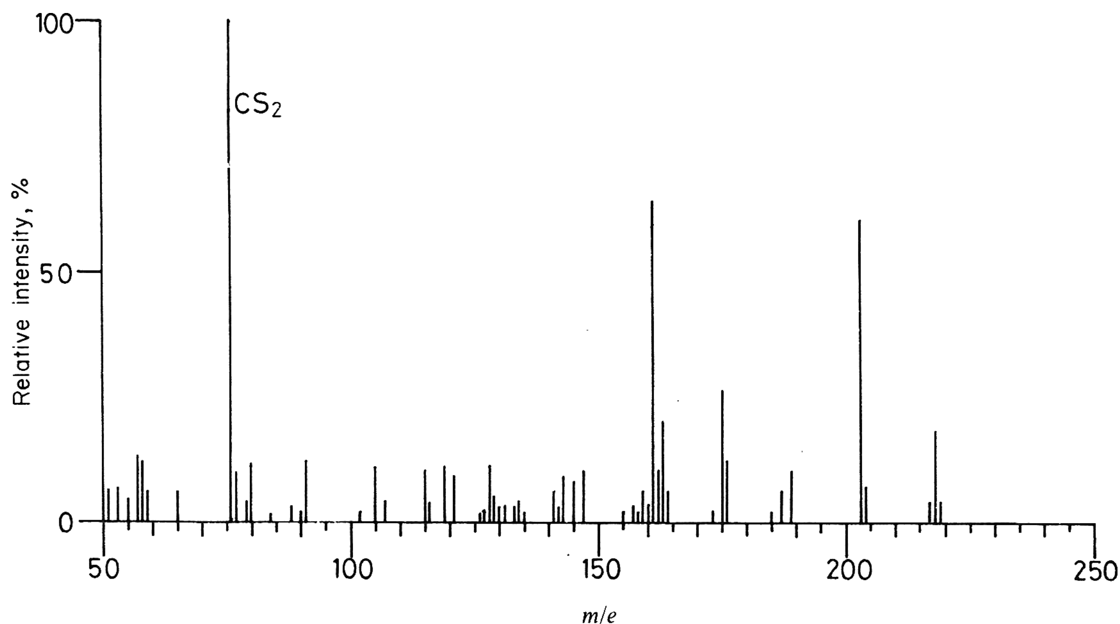

Fig. 2. Mass spectrum of the major hepatic metabolite of BHT (peak I in Fig. 1) in male rats.

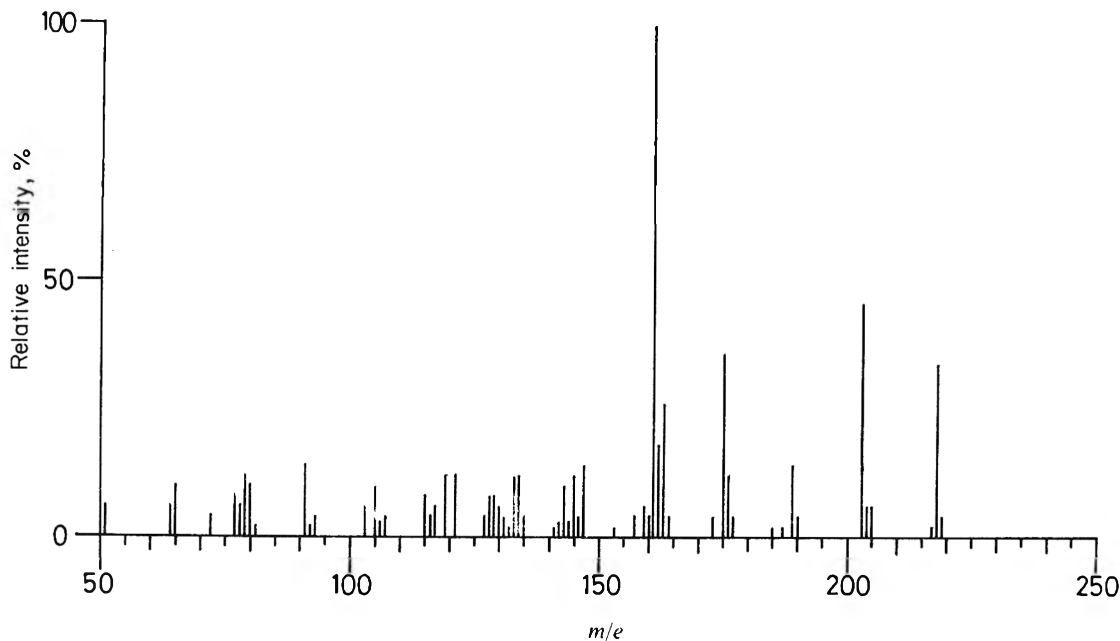

Fig. 3. Mass spectrum of authentic 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone. The gas-chromatographic retention time of this compound was the same as that of the major hepatic metabolite of BHT under the same conditions.

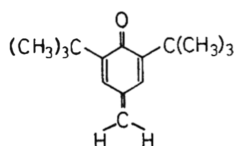

Fig. 4. Chemical structure of 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone.

2,6-di-*tert*-butylcyclohexa-2,5-dienone and 4-hydroperoxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dienone are produced by liver microsomes *in vitro* (Chen & Shaw, 1974; Shaw & Chen, 1972), but these metabolites were not detected as the corresponding quinone methides. The mass spectrum of an unidentified minor hepatic metabolite of mol wt 206 is reproduced in Fig. 5.

This work demonstrates that while BHT was deposited unchanged in several organs following its ingestion by rats, the corresponding quinone methide was present in the liver. Lowenthal & MacFarlane (1965) reported the anticoagulant activity of some substituted benzoquinones, the =O of which provides an electron pattern like the $=CH_2$ of the quinone methide. We should like to establish, therefore, whether rats are rendered haemorrhagic by this quinone methide.

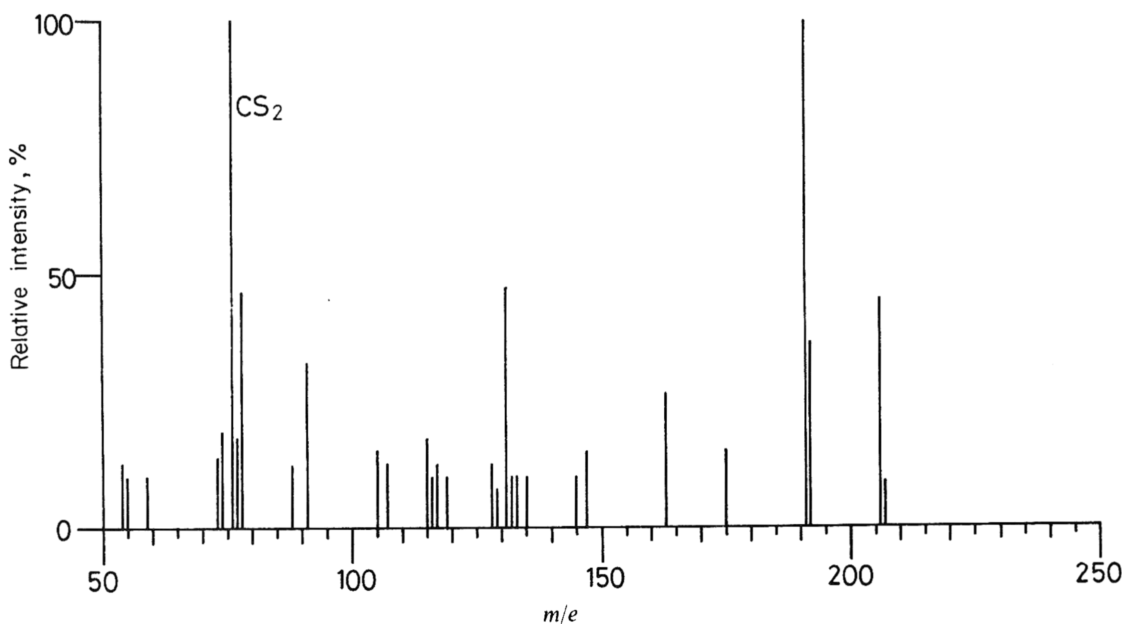

Fig. 5. Mass spectrum of the minor hepatic metabolite of BHT in male rats (peak II in Fig. 1).

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CONSUMPTION OF TOXIC RICE OIL BY 'YUSHO' PATIENTS AND ITS RELATION TO THE CLINICAL RESPONSE AND LATENT PERIOD

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Abstract—The amounts of toxic rice oil consumed individually by 141 patients with Yusho were estimated, taking into consideration their age and sex and the frequency of meals eaten at home. Since the total amount of oil consumed, the amount of oil consumed per kg per day, the amount of oil consumed during the latent period and the length of the latent period each showed a positively skew distribution, geometric means were calculated for all of them. The amounts of polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs) and polychlorinated quaterphenyls (PCQs) taken by the patients were, on average, 633, 3·4 and 596 mg, respectively, in total or 157, 0·9 and 148 μg/kg/day. The mean latent period was estimated to be 71 days, and the amounts of PCBs, PCDFs, and PCQs taken during the latent period were calculated to be, on average, 466, 2·5 and 439 mg, respectively, the smallest amounts taken by a patient during the latent period being estimated to be 111, 0·6 and 105 mg, and the smallest doses 29, 0·16 and 27 μg/kg/day, respectively. The clinical severity of the response showed a close positive correlation with the total amount of oil consumed but not with the amount of oil consumed per kg per day. The latent period and the amount of oil consumed per kg per day showed a highly significant negative correlation.

INTRODUCTION

A mass outbreak of food poisoning occurred in western Japan in 1968 following ingestion of a commercial brand of rice oil contaminated with polychlorinated biphenyls (PCBs) and related hydrocarbons (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972). The poisoning was named 'Yusho' (oil disease). Epidemiological proof of the cause of the epidemic depended on the demonstration of a dose-response relationship between the consumption of the toxic rice oil and the incidence of the poisoning or between the oil consumption and the clinical severity of the reaction. Therefore, a rough estimate of the quantity of the rice oil consumed by each patient and members of his or her family was made without regard to their age or sex, the overall food intake and the possible loss of the oil during and after cooking.

This demonstrated that both the incidence and the clinical severity of the poisoning correlated positively with the consumption of the rice oil (Kuratsune *et al.* 1972; Yoshimura, 1971). This survey of consumption also indicated that the average ingestion of PCBs, calculated as Kanechlor 400, was about 2 g per patient and the minimum was about 0·5 g or about 70 μg/kg/day, assuming that the rice oil contained on average 2500 ppm PCBs (Kuratsune, 1972; Kuratsune *et al.* 1972; Yoshimura, 1971).

Subsequently, several requests have been made to us, mainly from abroad, for more detailed data concerning the amount of the toxic rice oil ingested by the patients and their families. Moreover, recent investigations have revealed the important fact that the toxic rice oil contained considerably less PCB than was previously reported but relatively large amounts of other chlorinated hydrocarbons, such as polychlorinated dibenzofurans (PCDFs) (Nagayama, Masuda & Kuratsune, 1975) and polychlorinated quaterphenyls (PCQs) (Kamps, Trotter, Young, Carson, Roach, Sphon, Tanner & McMahon, 1978; Miyata, Murakami & Kashimoto, 1978). Consequently our original data were re-analysed, using somewhat different assumptions from those used in the former analysis, and taking the age, sex and food intake of the patients into consideration. The possibility of correlation between the individual oil intakes thus estimated and the clinical severity of the disease or its latent period was then investigated.

EXPERIMENTAL

Individual oil consumption. As already reported, patients developing Yusho had previously used either a canned rice oil or a bottled rice oil produced by one company (Kuratsune *et al.* 1972). In this study, only patients who were known to have used the canned rice oil, produced or shipped on 5/6 February 1968 were investigated, because the PCB content could be determined for this specific oil but not for the bottled ones because of a lack of samples. By interviewing the patients at home, the amount of the rice oil purchased, the amount of the oil remaining at home at the time of interview, the total time during which the oil was used, the time that elapsed before the consumers began to feel sick (the latent period) and the frequency with which they ate meals at home were determined.

From the total amount of the oil actually used by each household (T), the amount consumed by each member of the household was estimated, taking into account the person's sex and age and the frequency with which he or she ate meals at home and assuming

Table 1. *Daily consumption of fats and oils by males and females in Japan**

Age (yr)	Fats/oils intake (g/day)	
	M	F
0·5–1	3	3
1–11	10	10
12–14	15	20
15–19	25	20
20–39	20	20
40–69	15	15
70+	15	10

*National Food Constitution Table (Tezuka *et al.* 1970).

that in each case the consumption of this particular rice oil complied with the age- and sex-specific consumption of fats and oils (Fn) calculated for average Japanese in the National Food Constitution Table (Tezuka, Takai, Ikegami, Otani, Miyazaki, Tsutsumi, Matsumi & Nishimura, 1970), as shown in Table 1.

The frequency with which each person ate at home had to be quantified for use in the estimation. Therefore, when breakfast, lunch or dinner had been eaten at home almost every day every week, two points were given for each type of meal, while one point was given when the meal was eaten at home three or four times per week and no point was given for a meal eaten out nearly every day. Thus, when a person had eaten breakfast at home almost every day every week, eaten lunch out nearly every day and eaten dinner at home almost every day, he was given four points. Using these meal frequency points (Mn), the amount of the rice oil (Rn) consumed by any individual (n) in a household was estimated as follows:

$$Rn = \frac{Mn\ Fn}{\Sigma(Mn\ Fn)} \times T.$$

The amount of the oil consumed during the latent period was determined by dividing Rn by the total number of days the oil was used and multiplying by the latent period.

Body weight. The body weight of the patients was not measured but was assumed to be equal to the national age- and sex-specific averages derived from the National Nutrition Survey, 1969 made by the Ministry of Health and Welfare.

Clinical severity. The clinical severity of the response of the patients examined in this study was that determined by dermatologists in the summer of 1969 (Toshitani & Kitamura, 1971). Grade 0 was used to designate a condition with physical complaints but without skin lesions, grade 1 a condition with pigmentation of the skin and the mucous membrane, grade 2 a condition with comedone formation, grade 3 a condition with some acne, and grade 4 a condition with extensive distribution of acneform eruptions.

Concentration of PCBs, PCDFs and other compounds in the rice oil. The PCB concentration of the canned rice oil involved was originally estimated from its organic chlorine content to be 2000–3000 ppm, as Kanechlor 400 (Tsukamoto, 1969). More recently, three samples of the oil analysed by Tsukamoto (1969) were reanalysed for PCB by the official standard analytical method newly established by the Ministry of Health and Welfare, Japan, and levels of 830, 900 and 1030 ppm PCBs, as Kanechlor 400, were demonstrated (Nagayama *et al.* 1975). These figures are much smaller than the earlier estimates but are considered to be more specific for PCBs. Therefore, in this study, the average of these figures (920 ppm) was taken to represent the PCB concentration of the oil. For PCDFs and PCQs figures of 5 ppm (Nagayama *et al.* 1975) and 866 ppm (Miyata *et al.* 1978), respectively, were used.

Statistical analysis. Normality of sets of data was examined by the χ^2 goodness of fit test and the test of skewness and kurtosis (Snedecor & Cochran, 1974). Significant difference among means was determined by a *t* test. The Spearman coefficient of rank correlation (Conover, 1971) was calculated to examine the relationship between the clinical severity expressed by the grading and each of the variables, such as the total amount of oil consumed, the amount of oil consumed per kg per day, the amount of oil consumed during the latent period, and the length of the latent period. The relationship between the latent period and the amount of oil consumed per kg per day was examined by calculating the ordinary correlation coefficient and regression equation.

RESULTS

For 141 patients (72 males and 69 females) from 35 households in which the canned rice oil had been used, it was possible to determine adequately the total amount of oil consumed and the amount of oil consumed per kg per day, but for seven of them the latent period could not be established. The sex and age distributions of these patients are shown in Table 2.

No significant difference between sexes was seen in the distributions of the total amount of oil consumed, the amount of oil consumed per kg per day, the amount of oil consumed during the latent period, and the latent period. Therefore, males and females were combined when the distributions of these variables were to be examined. Each of these variables showed a skew distribution. The tests of skewness and kurtosis indicated that all these distributions were positively skew with highly significant values of g_1 (coefficient of skewness) and g_2 (coefficient of kurtosis), but after logarithmic transformation they were practically normal except for the latent period, as confirmed by the test of fit (Table 3). Therefore, geometric means were calculated for these three items (Table 4). For the latent period, neither actual values nor log values

Table 2. *Age distribution of Yusho patients*

Age-group (yr)	No. of patients		
	M	F	M + F
0–9	14	10	24
10–19	17	12	29
20–29	9	14	23
30–39	16	13	29
40–49	5	10	15
50–59	6	6	12
60+	5	4	9
All ages	72	69	141

Table 3. *Test of normality of distributions of the amount of oil consumed and the latent period*

Parameter	Value	Goodness of fit		Test of	
		d.f.	χ^2	Skewness (g_1)	Kurtosis (g_2)
Total amount of oil consumed	Actual	4	17·21***	1·715**	3·063**
	Log	4	3·28	0·257	−0·409
Amount of oil consumed per kg per day	Actual	5	30·92***	1·686**	3·645**
	Log	4	6·07	0·001	−0·351
Amount of oil consumed during latent period	Actual	5	25·48***	1·566**	2·960**
	Log	4	2·03	0·031	−0·188
Latent period	Actual	6	29·36***	0·446*	−0·948**
	Log	4	23·69***	−0·242	−1·036**

The hypothesis of normality is rejected at the 5% (*), 1% (**) and 0·5% level (***).

approximated to normal but the latter values took a considerably smaller g_1 than did the actual values. Therefore, geometric means were also calculated for the latent period (Table 4).

The total amounts of oil consumed by each of the 141 patients ranged widely, from 195 to 3375 ml, with a mean of 688 ml. The amounts were smaller for the age-group 0–11 yr than for any of the older groups, both in males and females. The smallest figure, 195 ml, was for a girl aged 8, with clinical severity grade 1, while in adults the smallest was 220 ml for a man aged 44 with clinical severity grade 3. The amounts of oil consumed per kg per day also ranged quite widely, from 31 to 923 μl/kg/day, with a mean of 171·1 μl/kg/day, but they were largest for the age-group 0–11 yr, in contrast to the total amounts of oil consumed. The amounts of oil consumed during the latent period ranged from 121 to 1934 ml with a mean of 506·4 ml, thus showing considerably less variation than either the total amount of oil consumed or the amount consumed per kg per day.

The total amounts of PCBs, PCDFs and PCQs taken by patients were, on average, 633, 3·4 and 596 mg, respectively, while the amounts taken per kg per day were, on average, 157, 0·9 and 148 μg/kg/day, respectively. The amounts of PCBs, PCDFs and PCQs taken during the latent period were calculated to be, on average, 466, 2·5 and 439 mg, respectively.

The smallest amounts of oil consumed by patients before the onset of subjective symptoms were also investigated. Table 5 shows six cases, three of each sex, in which symptoms were apparent before the total intake exceeded 200 ml. It is notable that one of these males and all of the females were children. The smallest intake, 121 ml of oil, should have contained 111 mg PCBs, 0·6 mg PCDFs and 105 mg PCQs. It should also be noted that those who had consumed the least amounts of oil before the onset of symptoms were not necessarily those showing a low degree of clinical severity.

The smallest amounts of oil consumed per kg per day during the latent period (50 μl/kg/day or less) are shown in Table 6 for three males and three females. The estimated daily intakes of PCBs, PCDFs and PCQs in the smallest quantity of oil (31 μl/kg/day) were 29, 0·16 and 27 μg/kg, respectively, providing about 56 μg organic chlorine compounds/kg in total.

The frequency distribution of the patients by age, sex and severity of symptoms is shown in Table 7. Since no significant difference in the latter parameter was seen between sexes, both sexes were combined for examination of a possible difference between age groups. A fairly marked difference was seen between the 0–11-yr age-group and a combination of all the remaining groups, the proportion of the patients with grade 0 or 1 (light cases) being 38·5% in the 0–11-yr group and 18·3% in the group aged 12 yr and over (corrected $\chi^2 = 3·94$). The corresponding proportion for the 12–19 age-group was 7·4%, again significantly smaller than that of the 0–11-yr-olds (corrected $\chi^2 = 5·63$), but the proportions of the older age-groups, 20–44 and 45 and over were 18·3 and 28·6%, both being considerably smaller than that of the 0–11-yr age-group but not significantly so. All these findings suggest that there were more mild cases among the child patients than among the older ones.

Significant positive correlations were observed between the clinical severity of the response and the total amount of oil consumed for males, females, and males and females combined, as shown in Table 8. Slightly smaller but significant positive correlations were also observed between the clinical severity and the amount of oil consumed during the latent period. The correlations were larger for females than for males. Contrary to these findings, no significant correlation was seen either between the clinical severity and the amount of oil consumed per kg per day or between the clinical severity and the latent period. Similar results were obtained when analysis was limited to the adults aged from 20–44, excluding the younger and the older ones.

Significant negative correlations were seen between the latent period and the amount of oil consumed per kg per day for males, females, or males and females combined, as shown in Table 9. When males and females were combined and divided according to age, similar but slightly larger significantly negative correlations were seen for all the age-groups.

DISCUSSION

A very accurate quantitative determination of the toxic rice oil consumed by each patient was not feasible, but fortunately we could make a fairly accurate estimate of the amount of oil consumed by his or her household. From this household consumption of the oil, we tried at first to estimate the oil consumed by each member of the household by assuming that

Table 4. *Mean and range of the total amount of oil consumed, the amount of oil consumed per kg per day, the amount of oil consumed during the latent period and the latent period, calculated according to age-group and sex*

Age-group (yr)	No. of patients, mean and range	Oil consumption: Total (ml)			Daily (μl/kg/day)			During latent period (ml)			Latent period (days)		
		M	F	M + F	M	F	M + F	M	F	M + F	M	F	M + F
0–11	No.	15	11	26	15	11	26	14	10	24	14	10	24
	Mean*	415·4	431·4	422·1	263·9	284·5	272·4	393·8	277·9	340·5	97·2	53·8	76·0
	Range	216–1174	195–1688	195–1688	134–923	68–861	68–923	199–880	146–554	146–880	30–160	20–150	20–160
12–19	No.	16	11	27	16	11	27	15	10	25	15	10	25
	Mean*	745·0	801·8	767·7	179·5	190·5	183·9	587·7	644·7	609·9	66·5	76·8	70·5
	Range	306–1944	400–1944	306–1944	72–608	46–551	46–608	306–1934	314–1458	306–1934	30–150	30–135	30–150
20–44	No.	28	32	60	28	32	60	28	30	58	28	30	58
	Mean*	789·7	896·6	845·1	140·6	168·1	154·7	576·8	606·4	591·9	70·9	72·3	71·6
	Range	220–2881	288–3375	220–3375	32–436	47–480	32–480	121–1768	231–1134	121–1768	25–190	30–160	25–190
45+	No.	13	15	28	13	15	28	13	14	27	13	14	27
	Mean*	553·7	698·6	627·1	117·5	139·2	128·7	375·6	496·4	434·1	57·7	73·7	65·5
	Range	230–2161	324–2161	230–2161	31–351	50–379	31–379	170–853	295–1326	170–1326	30–135	30–160	30–160
All ages	No.	72	69	141	72	69	141	70	64	134	70	64	134
	Mean*	639·5	742·5	688·0	163·9	179·0	171·1	495·5	518·7	506·4	71·7	70·0	70·9
	Range	216–2881	195–3375	195–3375	31–923	46–861	31–923	121–1934	146–1458	121–1934	25–190	20–160	20–190

*Geometric mean.

Table 5. *Data for the six Yusho patients consuming the smallest quantities of oil during the latent period*

Patient	Sex	Age (yr)	Latent period (days)	Amount of oil consumed: During latent period: Total (ml)	Daily (μl/kg/day)	Overall total (ml)	Severity of clinical response (grade)
T.K.	M	42	30	121	70	565	3
R.K.	M	56	30	170	99	905	3
T.S.	M	2	105	199	146	360	2
M.S.	F	8	90	146	68	195	1
K.M.	F	6	20	161	423	1125	4
M.Y.	F	1	40	200	500	200	0

Table 6. *Data for the six Yusho patients consuming the smallest daily doses of oil during the latent period*

Patient	Sex	Age (yr)	Latent period (days)	Amount of oil consumed: During latent period: Total (ml)	Daily (μl/kg/day)	Overall total (ml)	Severity of clinical response (grade)
H.O.	M	56	135	235	31	348	3
M.S.	M	44	120	220	32	220	3
S.S.	M	30	120	309	44	309	2
K.O.	F	19	135	314	46	465	0
S.O.	F	23	135	314	47	465	1
F.O.	F	46	135	353	50	523	0

Table 7. *Numbers of patients of each age-group and sex showing different grades of clinical response*

Age-group (yr)	Sex	Severity of clinical response: Grade.... 0	1	2	3	4
0–11	M	1	4	9	1	0
	F	1	4	1	2	3
	M + F	2	8	10	3	3
12–19	M	0	0	2	8	6
	F	1	1	0	6	3
	M + F	1	1	2	14	9
20–44	M	0	4	4	10	10
	F	2	5	5	9	11
	M + F	2	9	9	19	21
45+	M	3	1	4	3	2
	F	2	2	5	3	3
	M + F	5	3	9	6	5
All ages	M	4	9	19	22	18
	F	6	12	11	20	20
	M + F	10	21	30	42	38

Table 8. *Correlation between the severity of the clinical response and the total and daily amounts of oil consumed and the latent period*

Independent variables tested	Sex	r*	No.	P
Clinical severity and	M	0·435	72	0·000
total amount of oil	F	0·544	69	0·000
consumed	M + F	0·500	141	0·000
Clinical severity and	M	−0·194	72	0·12
amount of oil consumed	F	0·176	69	0·15
per kg per day	M + F	−0·004	141	1·00
Clinical severity and	M	0·256	70	0·04
amount of oil consumed	F	0·395	64	0·002
during latent period	M + F	0·337	134	0·000
Clinical severity and	M	0·109	70	0·37
latent period	F	0·021	64	0·88
	M + F	0·070	134	0·42

*Spearman coefficient of rank correlation.

the consumption was proportional to the national average oil consumption matched to his or her age. However, this estimation was not possible because such age- and sex-specific national data on average oil consumption were not available. Therefore, an estimate was based on the assumption that the personal oil consumption by each member of a household was in proportion to the age- and sex-specific intake of fats and oils determined in the National Food Constitution Table (Tezuka *et al.* 1970).

This Table was based on a consideration of the National Dietary Allowance as well as of various data

Table 9. *Linear regression analysis: latent period against the amount of oil consumed per kg per day*

Sex	Age (yr)	Regression equation ($y=$)	Correlation coefficient	P
M	1–85	2·538–0·309X	−0·399	<0·001
F	1–75	3·141–0·579X	−0·680	<0·001
M + F	1–85	2·815–0·434X	−0·536	<0·001
	1–11	3·394–0·624X	−0·602	<0·005
	12–19	2·867–0·453X	−0·650	<0·001
	20–44	2·931–0·492X	−0·588	<0·001
	45–85	3·064–0·595X	−0·621	<0·001

Log-transformed values of the independent variables were used for the test.

on food consumption obtained by the National Nutrition Survey, the Food Balance Sheet, and other related surveys. The Table is widely used by dieticians for compiling menus for mass feeding. The figures listed in the Table may not represent the precise amounts of oils consumed by average Japanese by age and sex but may reflect fairly well their relative quantitative relationship, permitting us to use them for allotting the household oil consumption proportionally to the oil consumption of individuals. Since the oil consumption varies greatly according to age, the present estimate of personal oil consumption must be considerably more accurate than that in the previous study, in which age and sex differences were entirely neglected for the estimation. It should be admitted, however, that the present estimate is still, to some extent, limited in accuracy.

One of the most interesting findings of this study seems to be the fact that the clinical severity of the response correlated closely with the total amount of oil consumed but not at all with the amount of oil consumed per kg per day. This was an unexpected finding because we had thought that a better correlation would be given when the dose of the toxic rice oil was expressed per kg per day instead of as the total amount, an expectation strengthened by the fact that induction of drug-metabolizing enzymes with PCBs in rats largely depended upon the dose given per kg per day rather than upon the total dose (Tanaka & Komatsu, 1972).

Nevertheless, the current finding, while unexpected, was in line with some other correlations tested; both the subjective symptoms reported by the patients and their blood PCB levels correlated closely with the total amount of oil consumed but not with the intake of oil per kg per day (H. Hayabuchi, unpublished data 1979; T. Yoshimura, H. Hayabuchi & M. Kuratsune, unpublished data 1979). This finding cannot be explained with any confidence at present. It may be associated with the capacity of PCBs and related compounds, particularly those that are relatively highly chlorinated, to accumulate in the body more readily than most other toxic substances.

In this connection, it is notable that the toxic rice oil consumed by the patients contained little of the less-chlorinated biphenyls but a relatively large proportion of the highly chlorinated biphenyls and dibenzofurans which tend to accumulate in the body, because the oil had been contaminated with Kanechlor 400 previously used as a heat-transfer agent (Kuroki & Masuda, 1977; Masuda, Kagawa & Kuratsune, 1974; Nagayama, Kuratsune & Masuda, 1976; Tsukamoto, 1969). In contrast to the clinical severity of the response, the latent period showed a negative correlation with the amount of oil consumed per kg per day, a finding in accord with the principles of toxicology.

The 1232 mg constituting the patient's average total intake of PCBs, PCDFs and PCQs (633, 3·4 plus 596 mg) is considerably smaller than the figure of 2 g previously reported (Kuratsune, 1972; Kuratsune *et al.* 1972; Yoshimura, 1971), because the previously assumed concentration of PCBs was higher than that used in the present calculation and geometric means were calculated for the present study while arithmetic means were used in the previous study.

It was also reported in one of the previous papers (Kuratsune, 1972) that the smallest amount of PCBs taken by a patient before the onset of symptoms was estimated to be 70 μg/kg/day. This figure was for a 30-yr-old man, patient S.S. in Table 6, who had consumed the toxic rice oil for 120 days before the onset of symptoms. In the present study, he was estimated to have consumed a total of 79 μg PCBs, PCDFs and PCQs/kg/day. This figure is fairly close to the previous one, but is not the smallest of the figures obtained in the present study, the smallest being 56 μg/kg/day as mentioned.

Dermatologists reported that the dermal lesions of the children were generally milder than those of the adults (Goto & Higuchi, 1969; Toshitani & Kitamura, 1971) and our study also supported this. It is interesting that these children had actually consumed larger amounts of the oil per kg per day than had the adults. Two facts possibly relevant to this finding are that children, in general, are less susceptible than adults to acne and the total amounts of the oil consumed by the children were less than those consumed by the adults, as already mentioned.

The primary purpose of this paper is to provide data on the oil consumption of Yusho patients. Although these data may show some lack of scientific precision because of the inevitable difficulties presented by the study, they are worth reporting, since the Yusho episode is unique among the outbreaks of food poisoning experienced by man. It is desirable that this information be utilized, with appropriate caution, for a variety of purposes by those studying the wider implications of this episode.

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INDUCTION PAR LE LINDANE DES MONOXYGENASES MICROSOMALES DU FOIE CHEZ LE RAT: EFFETS D'UN REGIME HYPERLIPIDIQUE

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Résumé—De jeunes rats mâles Wistar reçoivent soit une alimentation normolipidique, soit un régime hyperlipidique (39%, essentiellement sous forme de saindoux). Après 2 semaines, chaque groupe est divisé en deux lots: témoin et traité au lindane. Le traitement par le pesticide consiste en l'addition de 60 ppm dans l'alimentation équilibrée et 93 ppm dans le régime gras; dans les deux cas, l'apport de lindane est 18 μg/Kcal. Le traitement dure 4 semaines. Le régime gras augmente dans les microsomes hépatiques les activités potentielles de l'hydroxylation de l'aniline, de la NADPH-cytochrome *c* réductase et la concentration du cytochrome *P*-450, exprimées par foie entier. Il abaisse la durée de sommeil provoquée par l'hexobarbital. Il modifie la composition en acides gras de la phosphatidylcholine microsomale; il diminue la proportion de l'acide palmitique et élève la teneur en acide stéarique. Le traitement au lindane accélère la *N*-déméthylation de l'aminopyrine mesurée *in vitro* et il élève la teneur en *P*-450. Il augmente de 32% la proportion de l'acide linoléique dans la phosphatidylcholine microsomale. Ce dernier effet du pesticide, déjà constaté par d'autres auteurs avec le phénobarbital et l'éthanol, est discuté.

L'ingestion de lindane supprime l'excès des poids du corps et du tissu adipeux dû au régime gras. Cet antagonisme résulterait peut-être d'une interaction entre les deux facteurs sur la sécrétion des catécholamines surrénaliennes. Le lindane diminue moins le temps de sommeil provoqué par l'hexobarbital quand le régime est riche en lipides. Ce résultat est contradictoire avec les effets de l'ingestion du pesticide et du régime sur les activités potentielles des monoxygénases microsomales. On discute de l'éventualité d'un manque d'apport de NADPH pour le métabolisme hépatique de l'hexobarbital chez le rat à la fois traité par le pesticide et soumis au régime gras.

Abstract—Two groups of young male Wistar rats were fed either with a normal or with a high-fat (essentially lard) diet (3·5 and 39%, w/w, respectively). After the first 2 wk, each group was equally subdivided into a control group and a group treated for the remaining 4 wk with lindane, 60 ppm (mg/kg diet) for the normal-fat group and 93 ppm for the high-fat group, providing 18 μg lindane/Kcal in each case. The high-fat diet increased the potential activities of aniline hydroxylase and NADPH-cytochrome c reductase and the cytochrome *P*-450 concentration of the whole liver, and decreased the hexobarbital sleeping-time. The fatty acid composition of microsomal phosphatidylcholine reflected a decrease in palmitic acid and an increase in stearic acid with the high-fat diet. Lindane treatment accelerated aminopyrine-*N*-demethylation, measured *in vitro*, and increased the *P*-450 content. The content of linoleic acid in microsomal phosphatidylcholine was increased by 32%, a finding already demonstrated by other authors using phenobarbital and ethanol as inducers. The increase in body weight and in adipose tissue due to the high-fat diet was suppressed by lindane intake. An interaction of these two factors on adrenal catecholamine secretion could explain this antagonism. The hexobarbital sleeping-time was lowered less by lindane in animals receiving the high-fat diet, a finding in opposition to both lindane and diet effects upon the potential activities of microsomal monooxygenases. It seems possible that in the rat fed both lindane and a high-fat diet, there may be a deficiency of NADPH for hepatic metabolism of hexobarbital.

INTRODUCTION

L'induction des monoxygénases microsomales du foie est l'un des effets biologiques les plus sensibles, sinon le plus sensible, à la contamination par les insecticides organochlorés (Fouts, 1970). Effet qui se manifeste, chez l'animal de laboratoire, pour des quantités de pesticide parfois proches de celles de l'environnement. Nous avons montré que l'addition de 20 ppm de lindane à la nourriture du rat accélère, à moyen terme, la *N*-déméthylation de l'aminopyrine et l'hydroxylation de l'aniline (Pélissier et Albrecht, 1976). La dose probablement efficace serait même inférieure à 5 ppm (Lowy, Albrecht, Pélissier et Manchon, 1977).

En accord avec Suschetet et Causeret (1974), nous pensons qu'il est intéressant en recherche toxicologique d'introduire une "dimension nutritionnelle". L'ac-

tivité des monoxygénases microsomales, et donc le métabolisme des substances xénobiotiques, dépend d'ailleurs des conditions de nutrition des animaux (Campbell et Hayes, 1974).

Le rôle des lipides dans le métabolisme des composés xénobiotiques a fait l'objet de nombreuses études puisque les monoxygénases sont, dans l'hépatocyte, liées à une structure membranaire, le réticulum endoplasmique (Wade et Norred, 1976). Cependant à notre connaissance, hormis le travail de Béraud, Gaillard et Derache (1975), la littérature ne signale pas de recherches en ce domaine portant sur l'influence de régimes fortement hyperlipidiques. Or, un apport alimentaire excessif en lipides est fréquent dans les pays occidentaux et pose des problèmes de santé publique: obésité, maladies cardiovasculaires... (Dupin, 1978).

D'autre part, le lindane, molécule liposoluble, est réparti et mis en réserve dans le tissu adipeux (Hayes, Dale et Lebreton, 1963). On peut alors concevoir que les effets biologiques de ce pesticide diffèrent selon la quantité de graisses corporelles. C'est pourquoi, dans la présente expérience, nous ajoutons le lindane non seulement à un régime équilibré mais aussi à un régime hyperlipidique.

Nous étudions, chez le jeune rat mâle, l'éventuelle interaction des effets du lindane et de la quantité de lipides alimentaires sur les microsomes hépatiques: activité potentielle des monoxygénases, durée du sommeil provoqué par l'administration d'hexobarbital (épreuve *in vivo*) et composition en acides gras de la phosphatidylcholine.

METHODES EXPERIMENTALES

Animaux et traitement. Nous recevons au sevrage à 18–20 jours des rats mâles Wistar U exempts d'organismes pathogènes spécifiques. Nous les acclimatons à notre animalerie pendant 1 semaine; ils reçoivent *ad lib.* une provende commerciale qui titre 20% de protéines et 4% de lipides. Le poids moyen des animaux après cette semaine est 74,5 ± 1,58 (32).

Nous répartissons ensuite les rats en deux groupes. Le premier reçoit une alimentation équilibrée, le second un régime hyperlipidique (Tableau 1). Nous préparons ces régimes au laboratoire. Pour les deux régimes, l'essentiel de l'apport lipidique est fourni par du saindoux et dans une moindre mesure par de l'huile de maïs (0,7% pour le régime équilibré; 1,1% pour le régime gras). La teneur en acides gras des régimes est indiquée aussi sur le Tableau 1. L'acide linoléique représente 3,3% des calories totales pour l'alimentation équilibrée et 4,9% pour le régime hyperlipidique.

Après 2 semaines (poids des 16 rats sur régime équilibré 139.5 ± 4.72 g et sur régime gras 151,8 ± 4.50 g), chaque groupe est divisé en deux lots témoins et traités au lindane. Le traitement par le pesticide, qui consiste en l'addition de 60 ppm dans l'alimentation équilibrée et 93 ppm dans le régime gras, dure 4 semaines. Dans les deux cas, l'apport de lindane est 0.018 mg/Kcal.

Après un jeûne d'une nuit, nous pesons puis tuons les rats par décapitation. Le foie, les reins et les graisses périgénitales sont prélevés, lavés et pesés. La quantité de lindane du foie est déterminée par chromatographie en phase gazeuse avec un détecteur à capture d'électrons (Lovelock et Lipsky, 1960).

Nous mesurons la teneur en lipides totaux du foie selon la méthode que décrivent Rose, Vaughan et Steinberg (1964). Nous préparons les microsomes hépatiques suivant le procédé que nous avons signalé (Albrecht, Pélissier, Manchon et Rospars, 1973) et nous en déterminons la teneur en protéines (Lowry, Rosebrough, Farr et Randall, 1951).

Acides gras des régimes et de la phosphatidylcholine microsomale. La composition en acides gras des régimes équilibré et hyperlipidique ainsi que de la phosphatidylcholine des microsomes est évaluée par la méthode de chromatographie en phase gazeuse que rapportent Metcalfe, Schmitz et Pelka (1966).

Activités enzymatiques des microsomes. Nous mesurons la *N*-déméthylation de l'aminopyrine, l'hydroxylation de l'aniline, la teneur en cytochrome *P*-450 et l'activité de la NADPH-cytochrome c réductase. Nous indiquons dans un article antérieur les références des méthodes dont nous nous sommes inspirés ainsi que les modifications que nous y avons apportées (Pélissier, Faudemay, Manchon et Albrecht, 1978).

Epreuve *in vivo*: Sur un autre lot de 32 rats (huit par groupe), nous déterminons le temps de sommeil provoqué par l'administration d'hexobarbital. Le barbiturique sodé, en solution à 2,5%, est injecté par voie intrapéritonéale, à la dose de 100 mg/kg rat. La durée du sommeil correspond à la période s'étendant entre la disparition du réflexe de posture et le redressement du rat placé en *decubitus* latéral; la température ambiante est 22 ± 1°C.

Analyse statistique. L'expérience est bifactorielle 2 × 2; elle permet l'étude des deux facteurs (lindane, régime), chacun étant à deux niveaux. Nous effectuons une analyse de variance (Schwartz, 1966) après avoir vérifié sa légitimité par le test de Bartlett (Snedecor et Cochran, 1967). L'organisation de l'expérience permet

Tableau 1. *Composition pondérale centésimale, teneur en acides gras et composition énergétique des régimes*

Composants	Régime équilibré	Régime gras
Composition (%)		
Protéines (caséine)	18,2	27,1
Lipides	3,5	39,3
Glucides assimilables	56,4	13,3
Eau	10,8	5,7
Ballast	5,7	6,3
Sels minéraux	4,9	7,6
Vitamines	0,46	0,74
Acides gras (% acides gras totaux)		
C14:0	1.7	1,6
C16:0	24,0	30,4
C18:0	9,1	17,7
C18:1	29,7	42,2
C18:2	35,9	7,2
C18:3	0,8	—
C20:4	—	—
Composition énergétique		
Kcal/100 g	330	515
% Kcal protidiques	22,0	21,1
% Kcal lipidiques	9,6	68,6
% Kcal glucidiques	68,6	10,4

d'appliquer la méthode des blocs complets et de diminuer ainsi au mieux la variance résiduelle (Lellouch et Lazar, 1974). Nous testons (i) l'absence d'interaction entre les deux facteurs, (ii) l'effet du lindane, et (iii) l'effet du régime.

Nous indiquons sur les tableaux la moyenne et l'estimation de son écart-type pour chaque groupe expérimental ainsi que la valeur de la variance résiduelle s^2 commune aux quatres groupes. Nous signalons la signification statistique des comparaisons après l'analyse de variance. Nous considérons qu'une différence est significative lorsque la probabilité (P) qu'elle soit nulle est $<0{,}05$ et très significative lorsque $P < 0.01$. Notre expérience étant plurifactorielle , s'il n'y a pas d'interaction significative entre les deux facteurs, pour tester l'effet de l'un, nous comparons la moyenne de deux groupes à la moyenne de deux autres groupes; par exemple, pour apprécier l'effet du lindane: (témoins, régime équilibré) et (témoins, régime hyperlipidique) *contre* (lindane, régime équilibré) et (lindane, régime hyperlipidique).

Pour le poids relatif du foie ou des reins, nous effectuons une analyse de covariance (Lellouch et Lazar, 1974). Toutes ces statistiques sont faites sur une calculatrice électronique (Programma 602) grâce à des programmes mis au point au laboratoire par R. Lowy (non publiés).

RESULTATS

Caractéristiques des animaux

Pour chaque groupe expérimental nous indiquons sur le Tableau 2 la quantité de lindane ingéré ainsi que la teneur en pesticide du foie. Les rats traités ingèrent en moyenne environ 1300 fois plus de lindane que les témoins. La concentration du pesticide dans leur tissu hépatique y est 50 fois plus forte. Nous remarquons que la quantité totale de lindane par foie correspond à peu près au millième de la quantité ingérée pendant toute la durée de l'expérience (4 semaines).

La prise de nourriture des rats au régime gras est moindre que celle des animaux au régime normolipidique mais leurs ingesta caloriques sont plus élevés (résultats non présentés). De sorte que, l'addition de lindane étant dans tous les cas de 0,018 mg/Kcal, les rats au régime gras ont davantage ($+20\%$; $P < 0{,}01$) ingéré de pesticide. Cependant, la teneur en lindane de leur foie n'est pas sensiblement différente de celle des animaux au régime équilibré.

Nous observons une interaction antagonique ($P < 0.01$) entre les effets du lindane et du régime gras sur le poids des rats au sacrifice (Tableau 3). Le lindane abaisse de 21% ($P < 0{,}01$) le poids des animaux au régime gras alors qu'il ne modifie pas significativement le poids des rats au régime normolipidique. Le régime gras élève de 18% ($P < 0{,}01$) le poids corporel des rats témoins mais il est sans effet apparent sur le poids des traités. Le lindane supprime donc l'excès de poids dû au régime hyperlipidique.

Le lindane et le régime gras augmentent ($P < 0{,}01$) le poids relatif du foie, respectivement de 23 et 25%; les effets des deux facteurs sont additifs. Le poids relatif des reins est accru de 24% ($P < 0{,}01$) par le traitement au pesticide; il n'est pas significativement modifié par le régime gras.

Ni le pesticide ni la quantité de lipides alimentaires n'influencent sensiblement la concentration des protéines totales hépatiques (Tableau 4). Le régime gras élève de 20% ($P < 0{,}01$) la teneur en lipides totaux du foie; le lindane est sans effet apparent.

Activité des microsomes hépatiques

Effets du lindane. L'ingestion de lindane produit une augmentation ($P < 0{,}01$) de la concentration et de la quantité totale par foie des protéines microsomales, respectivement de 10 et 16% (Tableau 4).

Les résultats du Tableau 5 montrent que la *N*-déméthylation de l'aminopyrine est accélérée ($P < 0{,}01$) par le traitement au pesticide; l'activité enzymatique est accrue de 73% par rapport aux protéines et de 92% par foie. L'hydroxylation de l'aniline est aussi augmentée; cependant, seule l'activité totale par foie est très significativement modifiée de $+35\%$.

Le lindane élève ($P < 0{,}01$) la biosynthèse nette du *P*-450 respectivement de 31 et 53% par rapport aux protéines et au foie (Tableau 5). Dans cette expérience, la NADPH-cytochrome *c* réductase n'est pas sensiblement induite par le pesticide; l'activité totale par foie tend seulement à l'augmentation ($P > 0{,}05$).

Les résultats du Tableau 6 montrent que l'ingestion de lindane modifie la composition en acides gras de la phosphatidylcholine des microsomes. Elle abaisse de 9% ($P < 0{,}05$) la proportion de l'acide stéarique et augmente de 32% ($P < 0{,}01$) celle de l'acide linoléique.

Effets du régime hyperlipidique. Le régime gras ne modifie pas significativement la concentration des protéines microsomales mais il augmente de 29% $P < 0{,}01$) leur quantité totale par foie (Tableau 4).

Les résultats du Tableau 5 indiquent que l'activité spécifique, rapportée aux protéines, de la *N*-déméthy-

Tableau 2. *Ingestion de lindane et teneur du foie en lindane*

	Lindane			
	Ingestion*		Teneur foie*	
Régime	μg/jour/rat	μg/4 semaines/rat	μg/g foie	μg/foie
Equilibré				
Témoin	0.826 ± 0.0205	23.13 ± 0.574	0.069 ± 0.0138	0.59 ± 0.121
Lindane	1137 ± 26.0	31 836 ± 728	3.70 ± 0.331	36.0 ± 3.62
Hyperlipidique				
Témoin	1.073 ± 0.0262	30.04 ± 0.734	0.057 ± 0.0147	0.66 ± 0.184
Lindane	1363 ± 45.8	38 164 ± 1282	2.98 ± 0.404	35.9 ± 5.53

*Moyenne, écart-type sur la moyenne (huit animaux/groupe).

Tableau 3. *Poids du rat et poids du foie, des reins et des graisses périgénitales*

Régime	Poids corporel† ($n = 8$) (g)	Poids† Du foie ($n = 8$) g	g/100 g rat	Des reins ($n = 8$) g	g/100 g rat	Des graisses ($n = 4$) g	g/100 g rat
Equilibré							
Témoin	240,9 ± 6,92	8,68 ± 0,307	3,60 ± 0,115	2,191 ± 0,0567	0,909 ± 0,0567	2,221 ± 0,0601	0,871 ± 0,0235
Lindane	243,6 ± 7,85	9,98 ± 0,403	4,10 ± 0,149	2,70 ± 0,207	1,11 ± 0,765	3,20 ± 0,617	1,29 ± 0,249
Hyperlipidique							
Témoin	283,9 ± 7,71	11,84 ± 0,963	4,17 ± 0,305	2,594 ± 0,0835	0,914 ± 0,0265	3,76 ± 0,630	1,33 ± 0,223
Lindane	224 ± 11,6	12,14 ± 0,667	5,42 ± 0,267	2,56 ± 0,205	1,142 ± 0,0823	1,70 ± 0,333	0,720 ± 0,141
s^2 ...	662,6	2,561	0,2626	0,1660	0,01856	0,6318	0,07519
Comparaisons‡							
Effet lindane	a NS	NS	**	NS	**	a NS	a NS
	b *					b **	b **
Effet régime	c **	**	**	NS	NS	c *	c *
	d NS					d NS	d NS
Interaction	**	NS	NS	NS	NS	*	*

†Moyenne, écart-type sur la moyenne (nombres d'animaux indiqués), et valeur de la variance résiduelle (s^2).
‡Comparaisons par rapport à (a) régime équilibré, (b) régime hyperlipidique, (c) rats témoins et (d) rats traités au lindane: * = $P \leqslant 0{,}05$; ** = $P \leqslant 0{,}01$; NS = $P > 0{,}05$.

Tableau 4. *Protéines et lipides du foie des rats nourris avec des régimes contenant lindane*

Régime	Protéines† Totales (mg/100 mg foie)	Microsomales mg/100 mg foie	Microsomales g/foie	Lipides totaux† (mg/100 mg foie)
Equilibré				
Témoin	19.5 ± 1.02	3.53 ± 0.156	0.308 ± 0.0172	4.69 ± 0.227
Lindane	21.2 ± 1.13	3.89 ± 0.236	0.376 ± 0.0225	4.90 ± 0.201
Hyperlipidique				
Témoin	20,53 ± 0,960	3,49 ± 0,196	0,417 ± 0,0440	5,80 ± 0,244
Lindane	19,2 ± 1,28	3,87 ± 0,191	0,463 ± 0,0409	5,72 ± 0,265
s^2...	6,997	0,1429	0,003195	0,2858
		Comparaisons‡		
Effet lindane	NS	**	**	NS
Effet régime	NS	NS	**	**
Interaction	NS	NS	NS	NS

†Moyenne, écart-type sur la moyenne (huit animaux/group), et valeur de la variance résiduelle (s^2).
‡Comparaisons: ** = $P \leq 0.01$; NS = $P > 0.05$.

lation de l'aminopyrine ou de l'hydroxylation de l'aniline ne paraît pas dépendre significativement de la teneur en lipides du régime. Cependant, les activités totales par foie sont accrues ($P < 0{,}01$), respectivement de 31 et 42% par le régime gras.

De même, la teneur en P-450/100 mg de protéines n'est pas sensiblement modifiée mais la quantité totale de ce cytochrome par foie est augmentée de 36% ($P < 0{,}01$; Tableau 5).

L'activité spécifique de la NADPH-cytochrome *c* réductase n'est pas non plus significativement dépendante de la quantité de lipides alimentaires alors que l'activité totale par foie est élevée de 42% ($P < 0{,}01$).

Nous remarquons sur le Tableau 6 que le régime gras abaisse de 20% ($P < 0{,}01$) la teneur en acide palmitique de la phosphatidylcholine et qu'il augmente de 20% ($P < 0{,}01$) la proportion de l'acide stéarique.

Interaction des effets lindane × régime gras

Dans cette expérience, il n'y a pas d'interaction significative entre les effets des deux facteurs sur l'activité potentielle des monoxygénases et sur la composition en acides gras de la phosphatidylcholine microsomale. En revanche, nous observons une interaction antagonique ($P < 0{,}01$) pour l'épreuve *in vivo* (Tableau 5). Le régime gras diminue de 12% ($P < 0{,}05$) la durée du sommeil provoqué du rat témoin alors qu'il la prolonge de 13% ($P < 0{,}05$) chez l'animal traité au pesticide. Le lindane abaisse de 41% ($P < 0{,}01$) le temps de sommeil provoqué du rat au régime normolipidique mais la diminution n'est que de 24% ($P < 0{,}01$) chez l'animal au régime gras. En conclusion, le lindane diminue moins le temps de sommeil provoqué par l'hexobarbital lorsque le régime est hyperlipidique.

DISCUSSION

Dans une récente revue générale Wade et Norred (1976), rappellent les principaux faits expérimentaux établissant la nécéssité des lipides membranaires pour le métabolisme des composés xénobiotiques. Du point de vue nutritionnel, outre l'apport énergétique, les lipides apparemment nécessaires sont seulement les acides linoléique (C18:2) et linolénique (C18:3) que l'on qualifie d'ailleurs d'acides gras indispensables. Selon Norred et Wade (1972), l'addition de 3% d'huile de maïs, riche en linoléate, à un régime sans graisse, augmente la biosynthèse nette du P-450 et accélère les métabolismes de l'éthylmorphine, de l'hexobarbital et de l'aniline. Caster, Wade, Greene et Meadows (1970) considèrent que 3% des calories sous forme de linoléate dans un régime équilibré constituent un apport alimentaire optimal pour le métabolisme des substances xénobiotiques.

Béraud *et al.* (1975) constatent qu'un regime à 30% de lipides (huile d'arachide) augmente spécifiquement, après 21 jours, les métabolismes de l'aminopyrine et de l'aniline chez la ratte Sprague-Dawley. Dans notre travail, le régime gras n'a pas, chez le rat mâle Wistar, d'effet significatif sur les activités enzymatiques et sur la concentration du P-450, rapportées aux protéines. Cependant, il faut noter que les pourcentages d'augmentation de ces paramètres par foie entier, sous l'influence du régime gras (P-450 +36%; NADPH-cytochrome *c* réductase +42%; hydroxylation de l'aniline +42%) sont supérieurs à ceux du poids du foie (+29%) et de la quantité totale de protéines microsomales (+29%). Le régime gras réduit d'ailleurs de 12% le temps de sommeil provoqué par l'hexobarbital. Nos résultats ne sont donc pas en contradiction avec ceux de Béraud *et al.* (1975) mais ils sont moins nets. Outre les différences de race et de sexe des animaux, nous pensons que le moindre effet de notre régime gras sur les enzymes microsomales pourrait s'expliquer si l'on compare les compositions en lipides des deux régimes, particulièrement la teneur en acide linoléique. Le régime utilisé par Béraud *et al.* (1975) titre 30% de lipides sous forme d'huile d'arachide. Notre régime comporte 39% de lipides provenant essentiellement de saindoux. Nous avons calculé que le régime de Béraud *et al.* (1975) apporte environ deux fois plus d'acide linoléique que le nôtre: 5,5% (w/w) au lieu de 2,8%. Si notre hypothèse est exacte, elle

Tableau 5. *Activités enzymatiques des microsomes des rats nourris avec des régimes contenant du lindane*

Paramètre	Activité enzymatique†					Comparaisons‡		
	Régime équilibré		Régime hyperlipidique					
	Témoin	Avec lindane	Témoin	Avec lindane	s^2	Effet lindane	Effet régime	Interaction
N-Déméthylation de l'aminopyrine:								
nmol aminoantipyrine/min/100 mg protéines	9,9 ± 2,23	16,4 ± 2,09	10,9 ± 1,59	19,6 ± 4,26	17,22	**	NS	NS
nmol aminoantipyrine/min/foie	28,6 ± 5,06	59,6 ± 6,41	41,1 ± 3,09	75,0 ± 8,16	129,5	**	**	NS
Hydroxylation de l'aniline:								
nmol *p*-aminophénol/min/100 mg protéines	25,2 ± 2,74	31,3 ± 1,55	29,2 ± 1,30	31,5 ± 3,71	42,38	NS	NS	NS
nmol *p*-aminophénol/min/foie	76,7 ± 8,84	117,1 ± 8,79	123,1 ± 15,7	151,5 ± 27,6	1070	**	**	NS
Cytochrome*P*-450:								
nmol/100 mg protéines	67,0 ± 5,31	82,4 ± 3,30	67,4 ± 4,07	93,7 ± 7,48	240,3	**	NS	NS
nmol/foie	201,8 ± 9,23	310,9 ± 25,6	277,3 ± 27,1	422,0 ± 34,0	3617	**	**	NS
NADPH-cytochrome *c* reductase:								
μmol/min/100 mg protéines	5,10 ± 0,633	5,58 ± 0,706	5,66 ± 0,235	5,79 ± 0,602	1,957	NS	NS	NS
μmol/min/foie	15,2 ± 1,28	20,1 ± 1,80	23,6 ± 2,70	26,6 ± 3,66	231,0	NS	**	NS
Temps (min) de sommeil (effecteur hexobarbital)	25 ± 1	15 ± 0,8	22 ± 0,6	17 ± 0,7	3,810	a ** b **	c * d *	**

†Moyenne, écart-type sur la moyenne (huit animaux/groupe), et la valeur de la variance résiduelle (s^2).
‡Comparaisons par rapport à (a) régime équilibré, (b) régime hyperlipidique, (c) rats témoins et (d) rats traités au lindane: * = $P \leqslant 0,05$; ** = $P \leqslant 0,01$; NS = $P > 0,05$.

Tableau 6. *Teneur en acides gras des régimes et de la phosphatidylcholine des microsomes du foie des rats*

Acide gras	Acides gras individuels (mg/100 mg acides gras totaux)						s^2	Comparaisons‡		
	Régime équilibré			Régime hyperlipidique						
		Foie des rats†			Foie des rats†					
	Régime	Témoin	Lindane	Régime	Témoin	Lindane		Effet lindane	Effet régime	Interaction
C14:0	1,7	—	—	1,6	—	—	—	—	—	—
C16:0	24,0	40,2 ± 3,38	39,9 ± 1,57	30,4	32,66 ± 0,598	31,8 ± 2,11	15,12	NS	**	NS
C18:0	9,1	32,6 ± 3,11	31,17 ± 0,538	17,7	40,7 ± 1,35	35,8 ± 2,63	7,220	*	**	NS
C18:1	29,7	11,03 ± 0,627	10,44 ± 0,907	42,2	12,28 ± 0,913	10,75 ± 0,515	1,366	NS	NS	NS
C18:2	35,9	7,06 ± 0,656	8,19 ± 0,266	7,2	6,33 ± 0,336	9,5 ± 1,35	2,045	**	NS	NS
C18:3	0,8	2,41 ± 0,720	2,55 ± 0,297	—	3,38 ± 0,553	2,67 ± 0,525	1,065	NS	NS	NS
C20:4	—	6,4 ± 1,79	7,0 ± 1,54	—	4,6 ± 0,109	7,13 ± 0,648	6,689	NS	NS	NS

†Moyenne, écart-type sur la moyenne (huit animaux/groupe), et valeur de la variance résiduelle (s^2).
‡Comparaisons: * = $P < 0,05$; ** = $P < 0,01$; NS = $P > 0,05$.

signifie que les régimes hyperlipidiques augmentent l'activité des monoxygénases microsomales dans la mesure où ils élèvent l'apport en acide linoléique.

Le régime hyperlipidique augmente le poids corporel ainsi que celui du tissu adipeux. L'ingestion de lindane supprime ces excès de poids dûs au régime gras. Nous pensons qu'il pourrait s'agir là d'un effet consécutif à l'action neurotrope du pesticide. La stimulation de l'hypothalamus augmente la sécrétion de l'adrénocorticotrophine (ACTH) hypophysaire. On sait que l'ACTH, en activant la triglycéride-lipase ("lipase hormonosensible") accroît la lipolyse dans le tissu adipeux (Vaughan, 1964). L'ACTH agit aussi sur les surrénales. Les catécholamines de la médullosurrénale (Vaughan, 1964) et les glucocorticoïdes du cortex (Fain, Scow et Chernick, 1963) sont eux-mêmes des activateurs de la triglycéride-lipase. Or, dans un travail en cours, J. Roffi (communication personnelle, 1978) mesure les teneurs en catécholamines des surrénales chez des rattes Sherman traitées pendant 5 mois par des régimes comportant 60 ppm de lindane. L'alimentation est soit équilibrée, soit hyperlipidique (45% de saindoux plus 1% d'huile de noix). Il constate une interaction antagonique entre les effets du pesticide et du régime sur l'adrénaline surrénalienne: l'insecticide n'a pas d'effet significatif sur la quantité d'adrénaline lorsque le régime est équilibré alors qu'il la diminue avec le régime gras. Une moindre teneur en adrénaline surrénalienne résulte soit d'un accroissement de la sécrétion de l'hormone à partir de la médullosurrénale soit d'une diminution de sa synthèse. Dans le premier cas, l'adrénaline libérée agit sur la triglycéride-lipase de l'adipocyte et ainsi augmente la lipolyse. Si nous rapprochons nos résultats de ceux de J. Roffi, nous comprenons alors pourquoi le poids corporel et le poids du tissu adipeux sont très sensiblement diminués chez les rats traités et nourris avec le régime hyperlipidique. D'autre part nous comprenons aussi pourquoi il n'y a pas d'effet du lindane lorsque le régime est équilibré puisque nous venons de voir que la teneur en adrénaline de la surrénale des rats traités n'est pas différente de celle des témoins. Il resterait toutefois à prouver l'augmentation des catécholamines circulantes chez les animaux à la fois traités par le lindane et nourris au régime gras.

Par cette expérience, nous confirmons que l'ingestion de 18 μg lindane/Kcal de nourriture pendant 4 semaines, induit les monoxygénases du foie chez le jeune rat mâle. Nous avons déjà signalé que l'induction enzymatique par le pesticide s'accompagne d'une augmentation de la teneur en phosphatidylcholine (Pélissier *et al.* 1978). Nous constatons que, non seulement, la phosphatidylcholine est en plus grande quantité dans les microsomes du foie des rats traités, mais que le lindane modifie sa composition chimique: la proportion de l'acide linoléique est fortement augmentée. A notre connaissance, la littérature ne signale pas de travaux en ce domaine portant sur les insecticides organochlorés. Mais Davison et Wills (1974a) rapportent que le phénobarbital élève la proportion de cet acide, et Joly, Hetu, Mavier et Villeneuve (1976) remarquent un même effet dû à l'ingestion d'éthanol. Ainsi, trois inducteurs des enzymes microsomales (lindane, phénobarbital et éthanol) ont la même propriété d'augmenter la proportion de l'acide linoléique de la phosphatidylcholine microsomale. D'autre part, Grinna (1977) montre que cette proportion diminue avec l'âge des rats. Or on sait que généralement la capacité de métaboliser les composés xénobiotiques, et encore plus de "répondre" à l'induction, diminue quand les animaux vieillissent. Cet ensemble de faits expérimentaux nous amène à partager l'opinion de Davison et Wills (1974a) lorsqu'ils écrivent: "des espèces spécifiques de phosphatidylcholine (ou de phosphatidyléthanolamine) contenant de l'acide linoléique en position β seraient nécessaires pour la formation de la membrane microsomale et seraient associées aux protéines pendant le processus d'induction". L'acide linoléique, ou peut-être tout autre acide de sa famille ène ω-6, jouerait alors un rôle primordial dans l'induction des monoxygénases. Rôle que l'on peut rapprocher de l'effet "permissif" des acides gras de l'huile de hareng et du sitostérol oxydé que proposaient Marshall et McLean (1971). L'augmentation de la proportion de l'acide linoléique dans les microsomes hépatiques est-il un phénomène général lors de l'induction des enzymes liées au cytochrome P-450? A l'exclusion peut être de l'induction par les hydrocarbures polycycliques (Davison et Wills 1974a,b).

Dans ce travail, nous constatons une interaction entre les effets du régime gras et du lindane sur le temps de sommeil provoqué par l'administration d'hexobarbital. D'après nos résultats concernant les activités potentielles des monoxygénases microsomales, nous pouvions nous attendre à ce que le temps de sommeil provoqué par l'hexobarbital soit particulièrement diminué chez le rat traité nourri au régime gras. En effet, *in vitro*, les activités par foie entier sont accrues et par l'insecticide et par le régime. Or le régime hyperlipidique atténue l'effet du lindane sur la durée de sommeil provoqué. Il y a donc discordance des résultats *in vivo* et *in vitro*. Nous pensons qu'*in vivo* l'apport de NADPH pourrait être en quantité limitante lors de la biotransformation de l'hexobarbital chez le rat à la fois traité au lindane et nourri au régime gras. En effet, il est connu que les régimes hyperlipidiques abaissent le rapport NADPH/NADP cytoplasmique (Griglio, Goranov, Lavau et Lowy, 1969). De plus, on sait depuis peu que le métabolisme du lindane nécessite une forte demande en NADPH: son oxydation en hexachlorocyclohexanol (Stein, Portig et Koransky, 1977) et sa déshydrogénation en hexachlorocyclohexène (Chadwick, Chuang et Williams, 1975) sont des réactions microsomales dépendantes du NADPH et du P-450. C'est ainsi que les effets des substances xénobiotiques sur la cellules intacte pourraient différer de ceux que l'on observe *in vitro*. Il faut tenir compte *in vivo* de la relation réciproque des métabolismes intermédiaire et microsomal. Il s'agit là d'un point qui, semble-t-il, n'a retenu l'attention que de quelques groupes de chercheurs (Lowy et Derache, 1974; Pélissier, Manchon, Collomb et Albrecht, 1973; Schenkman, Cinti et Moldeus, 1973; Thurman, Marazzo et Scholz, 1974; Weigl et Sies, 1977).

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NITRITE SOURCES AND NITROSAMINE FORMATION *IN VITRO* AND *IN VIVO**

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Abstract—The ingestion by normal adults of a meal including vegetables rich in nitrate led to a rapid increase in the salivary nitrite concentration. This was followed by a fall towards the fasting nitrite levels, although the concentration in the saliva remained elevated for as long as 21 hr. Using foods as sources of nitrosatable amines, studies have been made both *in vitro* and *in vivo* on the formation of *N*-nitroso compounds under the conditions encountered in the human stomach, with special reference to the different thiocyanate concentrations in smokers and non-smokers. The nitrite level and pH of gastric contents increased markedly following the consumption of a meal containing nitrite, reaching maxima within about 45 min and then returning towards fasting levels. *N*-Nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine were the major volatile nitrosamines produced when foods were incubated with nitrite under acid conditions *in vitro*. At the nitrite concentrations likely to occur in the stomach, nitrosamine formation was reduced and was significantly lower in the absence of thiocyanate than in its presence at levels of 0·2–3 mM. Trace amounts of NPIP were detected occasionally in the gastric contents of volunteers after ingestion of homogenized foods containing nitrite. Both volatile and non-volatile *N*-nitroso compounds were obtained from a tobacco-smoke condensate following incubations *in vitro* simulating those likely to occur within the human stomach. Under the same conditions, *N*-nitrosation occurred in incubations containing the antidepressant nortriptyline, but this was greatly reduced in the presence of ascorbic acid.

INTRODUCTION

The nitrite concentrations in the saliva of a group of randomly selected individuals were found by Spiegelhalder, Eisenbrand & Preussmann (1976) to range from 0·14 μM to 0·43 mM, with an average value of 0·097 mM. Okabe (1973) reported even wider variations in a group of 200 Japanese subjects. Nevertheless, salivary nitrite levels have been found to increase significantly following the ingestion of nitrate-rich vegetables or their juices (Harada, Ishiwata, Nakamura, Tanimura & Ishidate, 1975; Ishiwata, Boriboon, Nakamura, Harada, Tanimura & Ishidate, 1975; Okabe, 1973; Spiegelhalder *et al.* 1976; Stephany & Schuller, 1975; Tannenbaum, Weisman & Fett, 1976). In general, the increase in nitrite concentration in the saliva correlated directly with the salivary nitrate content, although Spiegelhalder *et al.* (1976) observed a threshold intake of nitrate of about 54 mg, below which the salivary concentrations of nitrate and nitrite remained unchanged.

In the long-term feeding of nitrite and morpholine to rats, Shank & Newberne (1976) found that the nitrite concentration in the diet had a greater effect than the amine level on the formation of *N*-nitrosomorpholine and thereby on tumour induction, but Telling, Hoar, Caswell & Collings (1976) concluded that the concentration of dimethylamine or pyrrolidine in the diet had a greater influence on the formation of the corresponding nitrosamines in the rat stomach than did the level of nitrite in the drinking-water. The latter authors found no nitrosamine formation with amine levels of 100 ppm, although Shank & Newberne (1976) reported that levels of nitrite and the amine as low as 5 ppm were carcinogenic in a small proportion of treated rats.

In studies on the availability in foods of precursors of *N*-nitroso compounds, Walters, Newton, Parke & Walker (1974) detected four volatile nitrosamines, *N*-nitrosodimethylamine (NDMA), -diethylamine (NDEA), -pyrrolidine (NPYR) and -piperidine (NPIP), arising from the nitrosation of milk products. The nitrosatability of a secondary amine depends markedly on its basicity. The rate of nitrosation of piperazine (pK_a 5·57) is, for instance, no less than 185,000 times faster than that of piperidine (pK_a 11·2) under comparable conditions and at the optimum pH (Mirvish, 1975).

The series of *in vivo* and *in vitro* studies reported here were designed to relate these earlier findings to the normal patterns of food consumption by man and to assess, under conditions reflecting as closely as possible the actual situation in the consumer, the probable exposure to *N*-nitroso compounds resulting *in vivo* from dietary nitrite or nitrate. Changes in the nitrite concentration of human saliva as a result of ingestion of a meal consisting principally of salad veg-

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etables were determined, therefore. A further study examined the interactions of normal foodstuffs (as distinct from individual amines of unknown significance to the diet) during their retention in the human stomach. The possible influence of smoking and drugs on the *in vivo* formation of nitrosamines was also examined, in relation both to the provision of nitrosatable precursors and, in the former case, to the effect on levels of thiocyanate, a potent catalyst of secondary amine nitrosation (Boyland & Walker, 1974). Since tests involving tobacco-smoke condensates and drugs were not possible in man, *in vitro* conditions simulating those within the human stomach were used for this work. The nitrite level adopted was approximately the maximum detected in the stomach following ingestion of a meal containing nitrite, whilst the concentration of thiocyanate corresponded to the mean gastric level found in smokers.

EXPERIMENTAL

Effect of dietary nitrate on salivary nitrite levels. Each of a group of nine volunteers consumed nitrate-rich meals consisting of lettuce, beetroot, carrot, celery and radish to a total KNO_3 content of 90–115 mg (0·90–1·15 mmol), supplemented by cheese, bread and butter and an apple; water was provided *ad lib.* The test meals were taken on 3 days alternating with control days when no dietary regime was implemented, but the other foods and liquids taken were standardized as far as possible to avoid nitrate-rich components. Throughout the 5 days, samples of saliva of at least 3 ml were collected in beakers at regular intervals from the volunteers, following self-stimulated salivation, and were processed shortly afterwards.

Effect of dietary nitrite and gastric nitrite levels. Using Ryle's nasal tubes, samples of gastric juice were recovered from six student volunteers before and after consumption of a meal consisting of a fried egg (40 g), bread (32 g), butter (16 g), cheese (22 g), biscuits (17 g), milk (200 ml) and luncheon meat (80 g), the latter having been produced at BFMIRA without added nitrate. The calculated overall concentration of nitrite in the whole meal, excluding milk, was 0·83 mM.

Determination of thiocyanate levels. Samples of saliva were taken at intervals from 30 volunteers (15 smokers and 15 non-smokers) and samples of fasting gastric juice from 26 volunteers (six smokers and 20 non-smokers) for the determination of thiocyanate levels, by the method of Johnson (1916).

Determination of phenols. The level of total phenols were determined in gastric-juice samples taken at various intervals from one smoker and four non-smokers, using the Folin & Ciocalteu (1927) reagent.

Nitrosation of food amines in vitro. Using an MSE Atomix, meals similar to that used in the preceding experiment (including fried egg, bread, butter, cheese, biscuits, milk and luncheon meat) were homogenized in human gastric juice, the overall nitrite concentrations of the homogenates being adjusted to 0·46 mM (32 ppm as $NaNO_2$). After adjustment of the pH to 2·0 the homogenates were incubated for 3 hr at 37°C. In addition, various individual foods were homogenized separately and incubated with a 0·15-M concentration of nitrous acid. The whole-meal homogenate was also used to determine the effect of thiocyanate on nitrosamine formation. Any volatile nitrosamines formed were separated by distillation under reduced pressure by a method based on that of Telling, Bryce, Hoar, Osborne & Welti (1974).

Nitrosation of smoke condensate and nortriptyline hydrochloride in vitro. A tobacco smoke condensate was added to an HCl–KCl buffer of pH 2·0 to give a concentration of 1·0 g/100 ml. This was nitrosated under exhaustive and simulated gastric conditions, the nitrite concentrations being adjusted to either 0·14 M or 0·3 mM. Subsequently, incubations at 37°C were continued for 3 hr with agitation. Similarly, the antidepressant nortriptyline hydrochloride (3-(3-methylaminopropylidene)-1,2,4,5-dibenzocyclohepta-1,4-diene hydrochloride) at a concentration of 45·5 mg/100 ml was nitrosated under simulated gastric conditions in human gastric juice previously cleared by centrifugation and adjusted to pH 2·0, using an initial nitrite concentration of 0·3 mM. Any extractable *N*-nitroso compounds formed during the incubations were separated by repeated extraction into 1,2-dichloroethane.

Incubation of foods in vivo. Oral tubes of approximately 5-mm bore permitted both the entry and subsequent recovery of slurries of meals into and from the stomachs of volunteers after requisite time intervals. Experiments made with food slurries prepared with the addition of phenol red, a dye that is not absorbed from the stomach, indicated high recoveries (80–90%) of the food slurries by this method. The homogenates of meals consisting of egg, milk and luncheon meat with nitrite contents ranging from 0·46 to 0·77 mM (32–53 ppm) were of a consistency to permit passage along the oral tubes. After recovery of the slurries following residence within the human stomach, any volatile nitrosamines formed were separated by the technique used for the products of *in vitro* nitrosation.

Analytical methods

Determination of nitrite. After deproteinization with Carrez reagents (Adriaanse & Robbers, 1969) prepared free of nitrite, determinations were made by a method based on that of Shinn (1941).

Concentration and estimation of volatile nitrosamines. Volatile nitrosamines contained in distillates of *in vivo* or *in vitro* incubations were extracted into dichloromethane after addition of sodium chloride to the aqueous phase to give a concentration of 10% w/v. After careful concentration of the extracts in a Kuderna Danish evaporator placed in a water-bath thermostatically controlled at 55°C and subsequent transfer into hexane, individual volatile nitrosamines were detected and determined by gas chromatography using as a detector a high resolution Hitachi RMU7L mass spectrometer in the manner of Crathorne, Edwards, Jones, Walters & Woolford (1975). Volatile nitrosamines formed in incubations with smoke condensates were concentrated similarly and determined by a combined gas chromatograph–thermal energy analyser (Thermo Electron Corporation, Waltham, MA, USA).

Determination of extractable N*-nitroso compounds.* These compounds as a group were determined by the method of Downes, Edwards, Elsey & Walters (1976)

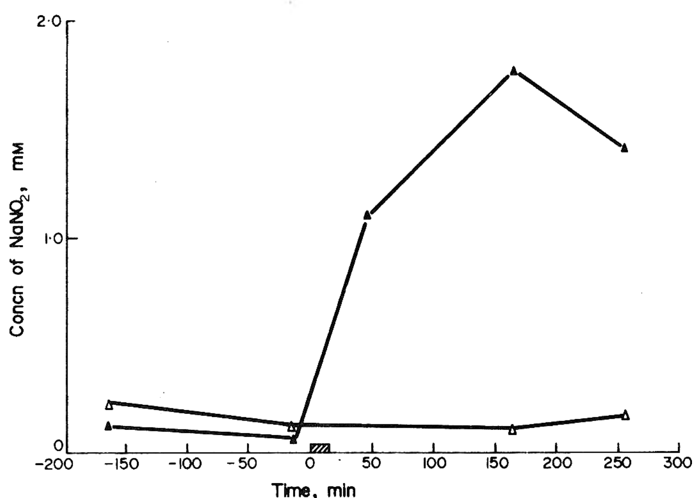

Fig. 1. Mean salivary nitrite levels before and after consumption (over the period ▨) of a salad-type meal containing 115 mg nitrate as KNO_3 (▲——▲) and on days when nitrate consumption was low (△——△).

using a chemiluminescence analyser. Differentiation from any inorganic or alkyl nitrites and *S*-nitrosothiols was accomplished through the use of acetic acid alone prior to the denitrosation of any *N*-nitroso compounds present, using hydrogen bromide in acetic acid.

RESULTS

Effect of dietary nitrate on salivary nitrite levels

In a group of normal volunteers, who showed a wide variation in initial salivary nitrite levels (mean ± SEM of 0·13 ± 0·0457 mM), consumption of salad-type meals rich in nitrate (90–115 mg or 0·90–1·15 mmol as KNO_3) was followed by a rapid rise in salivary nitrite levels (Fig. 1). Peak nitrite concentrations (1·77 ± 0·283 mM) were reached about 2 hr after completion of the meal, the differences between the peak and the basal values being highly significant. Approximately 21 hr after the test meal, on the intervening control days, the nitrite levels still showed a slight increase, although this decreased further during the following 2·5 hr. Individual variation in the salivary nitrite levels was generally small on the control days, when nitrate-rich dietary components were avoided as far as possible.

Effect of dietary nitrite on gastric nitrite levels

Following ingestion of a meal containing nitrite at an overall level of 0·83 mM, a rapid rise in mean pH and in gastric levels of nitrite took place, with maximum values for both occurring approximately 40 min after commencement of the meal (Fig. 2). Thereafter, the pH started to fall towards the average fasting value and there was a sharp decrease in the mean gastric nitrite level from about 60 min. The nitrite concentration in the fasting gastric juice of the subjects was 14 ± 8·17 μM (mean ± SEM).

Fasting levels of thiocyanate and phenols

The concentrations of thiocyanate in both the saliva and gastric juice of smokers were on average three or four times higher than those of non-smokers. In both groups, levels were lower in the gastric contents (0·48 ± 0·076 ($n = 20$) and 1·49 ± 0·094 mM ($n = 6$), respectively, for non-smokers and smokers) than in the saliva (1·82 ± 0·183 ($n = 15$) and 5·45 ± 0·190 ($n = 15$) respectively) presumably due to dilution by gastric juice. The difference between the thiocyanate levels in the saliva of smokers and non-smokers was maintained in repeated determinations throughout a day (Fig. 3).

In a limited study of the concentration of total phenols in gastric juice, analysis of 19 samples from four non-smokers gave values in the fasting gastric juice ranging from 1·2 to 4·3 mM, as phenol, with an average value of 2·06 mM. The corresponding range for five determinations on samples from a smoker was 1·5–3·5 mM, with an average of 2·64 mM. Thus on the basis of the small number of observations made, no significant difference was apparent between the two groups.

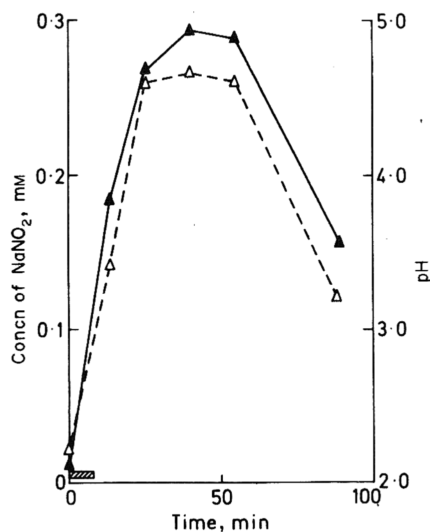

Fig. 2. Mean gastric nitrite concentrations (▲——▲) and pH values (△– – –△) following consumption (over the period ▨) of a meal containing 0·83 mM nitrite (58 ppm as $NaNO_2$).

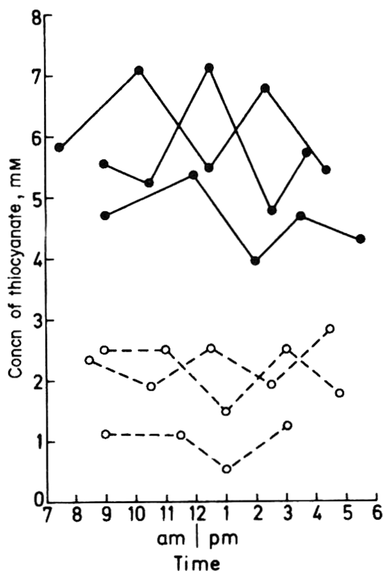

Fig. 3. Variation of salivary thiocyanate concentrations with time, over a working day, in three smokers (●——●) and three non-smokers (O---O).

Nitrosation of amines in vitro

Nitrosation of food amines. The predominant volatile nitrosamines arising from the deliberate nitrosation of foods, particularly those of dairy origin, by means of a high concentration of nitrous acid (0·15 M) were NPIP and NPYR. The maximum levels of each detected by gas chromatography coupled with high-resolution mass spectrometry were 83 and 42 mg/kg, respectively.

Table 1 illustrates the extent to which increasing the concentration of thiocyanate above that found in the fasting gastric juice of a smoker affects the average formation of NPIP and NPYR by a whole-meal slurry containing an overall nitrite concentration of 0·46 mM and incubated for 3 hr at pH 2·0 and 37°C. A significant difference ($P < 0·05$ by Student's t test) was noted between the formation of NPIP in the absence of thiocyanate and that in its presence at any level, but no significant variation was apparent within the range of thiocyanate concentrations tested (0·2–3·0 mM). NPYR production was very small throughout and no significant variations were noted.

The formation of NPIP and NPYR was determined also in slurries of meals consisting of luncheon meat, egg and milk (as used in *in vivo* studies) incubated for up to 3 hr at 37°C and pH 2·0. Nitrite was present to an overall concentration of 0·43 mM and thiocyanate was added to 1·2 mM. The formation of NPIP to a mean value of 6·7 mg/kg was apparent after incubation for 15 min and did not increase further when incubation was prolonged for up to 3 hr. NPYR formation was extremely small, ranging between 0·2 and 0·3 mg/kg.

Nitrosation of nortriptyline. The initial realistic nitrite concentration adopted (0·3 mM) fell rapidly within the first 30 min at 37°C and pH 2·0 to approximately 0·12 mM. Continued incubation resulted in concentrations of 0·07, 0·04, 0·03 and 0·02 mM after 60, 90, 120 and 180 min respectively. The residual nitrite

Table 1. *Effect of thiocyanate concentration on mean formation of volatile nitrosamines from food amines following incubation with 0·46 mM nitrite at pH 2·0 and 37 C*

Thiocyanate concn (mM)	Formation of volatile nitrosamines (µg/kg food) NPYR	NPIP
0	0·0	8·0
0·2	2·0	36
0·6	1·0	30
1·2	1·0	34
3·0	1·0	51

NPYR = *N*-Nitrosopyrrolidine
NPIP = *N*-Nitrosopiperidine

was removed with 45 mM-sulphamic acid before extraction of any *N*-nitroso compounds.

N-Nitroso compounds were formed in the presence and absence of nortriptyline hydrochloride, the additional extractable *N*-nitroso compounds attributable to the drug amounting to 31 µg as *N*-nitrosonortriptyline, only a small proportion of the total dose. The inclusion of ascorbic acid at a concentration of 5·0 mM in the incubations reduced the additional nitrosation by 75%.

Nitrosation of amines in a tobacco-smoke condensate. Exhaustive nitrosation of the condensate yielded 880 µg extractable *N*-nitroso compounds (as NPYR)/g condensate. This compared with an initial content of 1·2 µg (as NPYR)/g in the untreated smoke condensate. Of the *N*-nitroso compounds formed, 17% could be separated by distillation under reduced pressure from a solution of 10% w/v sodium chloride in the manner of Telling *et al.* (1974), the remaining 83% being non-volatile. The volatile nitrosamines, equivalent to 150 µg (as NPYR)/g smoke condensate were characterized, by gas chromatography coupled with the thermal energy analyser, as NDMA (35%) and NPYR (53%), with 12% being either lost or unaccounted for as a simple volatile compound.

The reduced nitrite concentration in the simulated gastric nitrosations led to only small recoveries of *N*-nitroso compounds extractable into dichloromethane. Of the total production of *N*-nitroso compounds (12 µg/g tobacco-smoke condensate expressed as NPYR), only about 10% was found in the fraction that distilled in steam under reduced pressure, with approximately 70% remaining non-volatile. Thus, 20% of the *N*-nitroso compounds produced at the lower nitrite concentration were either not recovered from the distillation process or were lost, presumably as a result of their lability.

Nitrosation of food amines in vivo

No NPIP was detected in slurries recovered on three separate occasions from the stomach of a smoker and a non-smoker 15 min after introduction of a homogenate of luncheon meat, egg and milk with an overall nitrite concentration of 0·46 mM. However, when similar homogenates with a mean nitrite concentration of 0·51 mM were retained in the human stomach for 30 min, NPIP was detected three times in the stomach contents from the smoker (at concentrations of 0·52, 0·27 and 0·54 µg/kg) and once in that

from the non-smoker (0·36 μg/kg) in seven tests on each subject. No NPIP was detected in the samples from either the smoker or non-smoker on the one occasion when it was possible to extend the time of incubation in the stomach to 60 min. No other volatile nitrosamines of the simple dialkyl type were identified in any of the samples.

Since the use of an oral tube for the introduction of the samples into the stomach and their removal from it might have reduced the availability of thiocyanate from the saliva, meals containing 0·49–0·64 mM (34–44 ppm) nitrite were eaten whole and as much as possible was recovered via the oral tube. Only 40–50% of the intake was recovered in this manner, compared with a mean recovery of 85% of the comminuted form, and neither NPIP nor NPYR was detected in any of the six samples analysed following recovery after both 30 and 60 min in the stomach.

DISCUSSION

The great individual variation in salivary nitrite concentrations reported by several workers was borne out by these studies. Okabe (1973) found nitrite levels ranging from 0·0014 to 2·9 mM in the saliva of 200 Japanese subjects, while values in the range 0·00014–0·43 mM, with an average of 0·097 mM, were obtained by Spiegelhalder *et al.* (1976), who concluded that differences in dental hygiene could not be the only cause for the large variations observed. The studies reported here provide a measure of the extent and duration of the rise in salivary nitrite concentration resulting from the consumption of a salad-type meal that could well be chosen by members of the general public.

In comparison with the levels observed on control days when the volunteers consumed other meals generally low in nitrate, the increases in salivary nitrite shortly after the ingestion of salads were highly significant. At the peak salivary nitrite concentration, the conversion factor obtained, namely an average increase of 98 ppm sodium nitrite in the saliva for each 100 mg potassium nitrate ingested was in reasonable agreement with the mean value of approximately 140 ppm $NaNO_2$/100 mg KNO_3 calculated from the data of Tannenbaum *et al.* (1976), whilst the conversion found by Spiegelhalder *et al.* (1977) was 20 ppm $NaNO_2$/100 mg KNO_3. Assuming a flow of saliva of 50 ml/hr, it is estimated that about 20 mg sodium nitrite would enter the stomach within 4–5 hr, after the ingestion of a nitrate-rich salad meal of the type described here. This compares with a value of approximately 40 mg nitrite resulting within the same period from the drinking of red-beet juice containing four times as much nitrate (Spiegelhalder *et al.* 1976), which suggests that nitrate contained in lettuce, beets etc. is extracted and utilized at least as readily as that swallowed in solution in an extract. The peak salivary concentration of nitrite occurred between 50 and 250 min after the commencement of the salad-type meals. Over a period of 30 min around the time of the observed peak nitrite concentration, a saliva flow of 50 ml/hr would introduce into the stomach approximately 3 mg sodium nitrite. Judging by the further *in vivo* studies conducted, the pH by that time could have already decreased towards the low fasting value of the stomach as a result of hydrochloric acid secretion to overcome the buffering action of the meal. This situation differs from that of the consumption of a meal containing nitrite itself, in which case the gastric nitrite concentration increases gradually, presumably as a result of its distribution amongst the stomach contents, and then reacts with phenolic, amino, sulphydryl and other groups over a short period as the pH falls as a result of hydrochloric acid secretion. The maximum level of nitrite observed in the stomach following its ingestion in a meal was in keeping with its distribution from the food throughout the stomach contents (about 1 litre in volume), suggesting that losses by absorption or by passage into the duodenum were small. In keeping with these results, positive indications of the formation of NPIP *in vivo* were not obtained with residence times of less than 30 min, by which time gastric pH values would presumably have been lowered by the renewed secretion of hydrochloric acid into the stomach.

The difference between the thiocyanate levels in the saliva of smokers and non-smokers observed by Densen, Davidow, Bass & Jones (1967) was confirmed and was also found in the gastric juice obtained from such groups, although the actual concentrations were lower in the gastric juice than in the saliva. Nevertheless, thiocyanate levels in the gastric juice of both smokers and non-smokers fell within the range considered by Boyland & Walker (1974) to be significant as a factor in the stimulation of the nitrosation of secondary amines at pH 3·0 and below. Phenols of various types have been considered either to inhibit (Challis, 1973) or to stimulate (Challis & Bartlett, 1975; Davis & McWeeny, 1977; Walker, Pignatelli & Castegnaro, 1975) the formation of nitrosamines from a secondary amine. Since, however, their levels in fasting gastric juice showed no difference between smokers and non-smokers, neither effect on nitrosation is more likely to occur in one group than in the other.

The NPIP and NPYR formed in foods in contact with nitrite may well not be the bases themselves but derivatives such as piperine, which can occur in peppers. Certainly the formation of these volatile nitrosamines from spices treated with nitrite is well authenticated (Gough & Goodhead, 1975; Sen, Miles. Donaldson, Panalaks & Iyengar, 1973) and the conversion of piperine to NPIP has been demonstrated by Lijinsky, Conrad & Van de Bogart (1972). At a nitrite concentration likely to be close to the maximum that could be produced in the stomach by dietary means, the formation of NPIP within a realistic residence time in that organ was approximately 10% of that produced during drastic nitrosation and was stimulated considerably in the presence of thiocyanate. Formation of NPYR, on the other hand, represented only a small proportion of the total produced under drastic nitrosation conditions, even when stimulated by thiocyanate, and this suggests that different pathways are operative in the production of these two volatile nitrosamines. At low pH values and nitrite concentrations, for instance, the nitrous acid route of nitrosation, in which the reaction rate is proportional directly to both the nitrite and hydrogen ion concentrations, is favoured (Mirvish, Sams & Hecht, 1977).

The reaction of the analgesics propoxyphene and norpropoxyphene with nitrous acid has been used for their simultaneous determination (Serfontein & de Villiers, 1976). At a nitrite concentration that could be achieved within the human stomach, nitrosation of the drug nortriptyline was apparent, although it involved only a small percentage of the total dose. To date, the possible carcinogenicity of *N*-nitrosonortryptyline has not been investigated. Nevertheless, its potential yield arising from *in vivo* nitrosation of the maximum recommended dose of nortriptyline is likely to be far greater than the amount of volatile nitrosamines ingested in the average British diet. The pronounced inhibitory action of ascorbic acid on the nitrosation of nortriptyline under conditions simulating those within the human stomach is encouraging in relation to the use of combined formulations designed to reduce or prevent nitrosamine formation *in vivo*.

The most likely major component of the non-volatile fraction of *N*-nitroso compounds produced by nitrosation of a tobacco-smoke condensate is *N*-nitrosonornicotine, arising from the nicotine present (Hoffmann, Hecht, Ornaf, Wynder & Tso, 1976). Currently, little if any information is available on the amount of nicotine entering the stomach of a smoker, although a quantity will be contained in swallowed saliva. Probably, therefore, the most important point brought out by this part of these studies is the much smaller amount of *N*-nitroso compound found in the untreated condensate itself compared with that produced in the presence of nitrite under simulated *in vivo* conditions.

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CONVERSION OF AFLATOXICOL TO AFLATOXIN B_1 IN RATS *IN VIVO* AND IN PRIMARY HEPATOCYTE CULTURE

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Abstract—The hepato-metabolic conversion of aflatoxicol to aflatoxin B_1 was studied by administering [^{14}C]aflatoxicol to the rat iv and to rat primary hepatocyte culture. Aflatoxicol was found to be rapidly metabolized to the parent toxin, aflatoxin B_1, both in the rat *in vivo* and in primary hepatocyte culture. The aflatoxin B_1 so-formed was confirmed by thin-layer chromatography, autoradiography and high-pressure liquid chromatography, and that formed *in vitro* was also confirmed by ultraviolet absorption spectroscopy, and by its mutagenic potency. In comparison to aflatoxin B_1, aflatoxicol showed a higher level of distribution to the tissues and a shorter plasma biological half-life. The biological potency of aflatoxicol and the correlation of its formation with species sensitivity to the carcinogenic effect of aflatoxin B_1 may be due to its ability to undergo oxidation to the parent toxin, aflatoxin B_1, allowing further bioactivation.

INTRODUCTION

Aflatoxin B_1 (AFB_1), a potent foodborne carcinogenic mycotoxin, is known to be biotransformed by various animal species into several metabolites with different toxicological characteristics (Campbell & Hayes, 1976). One of these metabolites is aflatoxicol (AFL), a reduced cyclopentenol product of AFB_1 whose formation is catalyzed by cytoplasmic reductase(s). AFL has been produced *in vitro* (Patterson & Roberts, 1971; Salhab & Edwards, 1977a; Schoenhard, Lee, Howell, Pawlowski, Libbey & Sinnhuber, 1976) and recently identified as an *in vivo* metabolite in rat plasma (Wong & Hsieh, 1978). Previous *in vitro* studies using liver homogenates have shown that AFL can be oxidized back to AFB_1 (Patterson & Roberts, 1972; Salhab & Edwards, 1977a) (Fig. 1). It has been suggested that the reversibility of conversion of AFL to AFB_1 may prolong cellular exposure to the toxic effects of AFB_1 (Loveland, Sinnhuber, Berggren, Libbey, Nixon & Pawlowski, 1977; Patterson & Roberts, 1972). Recent studies using liver homogenates have also suggested that the sensitivity of an animal species to the carcinogenic effect of AFB_1 can be directly correlated with hepatic activity for transforming AFB_1 to AFL (Hsieh, Wong, Wong, Michas & Ruebner, 1977). AFL is indeed the most carcinogenic (Schoenhard, 1974) and mutagenic (Wong & Hsieh, 1976) metabolite of AFB_1 so far isolated and identified. In the present study, the conversion of AFL to AFB_1 was studied under *in vivo* conditions and also in the primary hepatocyte cultures of the rat, a species highly sensitive to the carcinogenic effect of AFB_1.

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EXPERIMENTAL

Animals. Male Sprague–Dawley rats (Simonson Labs, Gilroy, CA) weighing 240–260 g and male New Zealand rabbits (4–5 months) were purchased from Animal Resource Services, University of California, Davis. The animals were fed Purina Laboratory Chow (Ralston Purina, St. Louis, MO) and tap water *ad lib.*

Chemicals. Non-radioactive AFB_1 was purchased from Calbiochem, La Jolla, CA. Ring-labelled [^{14}C]-AFB_1 was prepared from cultures of *Aspergillus parasiticus* ATCC 15517 supplemented with [1-^{14}C] acetate according to a previous procedure (Hsieh & Mateles, 1971). Purification of AFB_1 consisted of repeated thin-layer chromatography (TLC) on silica gel (Brinkman Instruments, Inc., Westbury, NY) developed with chloroform–acetone (9:1), followed by high-pressure liquid chromatography as described previously (Hsieh, Fitzell, Miller & Seiber, 1976). [^{14}C]AFL was produced by enzymatic conversion of [^{14}C]AFB_1 (Salhab & Edwards, 1977b) as summarized below. Four rabbits were killed, and their livers were excised and pooled for homogenization by Polytron® (Brinkman Instruments, Westbury, NY) in 2·5 volumes of ice-cold 0·25 M-sucrose solution containing 3 mM-$MgCl_2$ and 50 mM-HEPES buffer, pH 6·0. The homogenate was centrifuged at 12,000 g for 30 min, and the supernatant (S12) was centrifuged at 180,000 g for 1 hr. The final supernatant (S180), an equivalent of 300 mg pooled liver per ml of total volume, was supplemented with glucose-6-phosphate (6 mM) and $NADP^+$ (1 mM) and incubated for 30 min at 37°C in a CO atmosphere with [^{14}C]AFB_1 (5 μCi/0·5 mg). The aflatoxin metabolites were extracted several times using chloroform and chloroform–acetone (9:1). The nonpolar extracts were streaked on silica gel (Adsorbosil 1) TLC plates and developed with diethyl ether for the separation of AFL from AFB_1. Silica gel containing AFL was soaked in deionized water, macerated, and the metabolites were extracted several

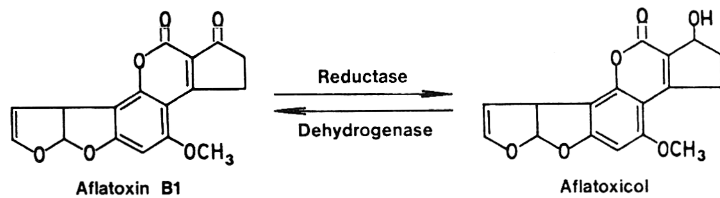

Fig. 1. Enzymatic interconversion of aflatoxin B_1 and aflatoxicol.

times with chloroform. AFL was purified on TLC plates (Merck Co., Darmstadt, Republic of Germany) developed with diethyl ether and was identified by TLC and by UV absorption spectrometry. Radiochemical purity of [^{14}C]AFB_1 and [^{14}C]AFL was confirmed by autoradiography, TLC and scintillation counting. The bioconversion of AFB_1 to AFL was consistently greater than 30% of the added AFB_1. Over 50% of the total AFL present was recovered in the final purification. The final [^{14}C]AFL substrate was diluted with unlabelled compound to a specific activity of 0·5 Ci/mol. Substrate purity was confirmed by TLC and autoradiography. All biochemicals were purchased from Sigma Chemical Co., St. Louis, MO. Authentic standards of AFB_1 metabolites including aflatoxin Q_1 (AFQ_1), aflatoxin P_1 (AFP_1), aflatoxin M_1 (AFM_1), aflatoxicol H_1 ($AFLH_1$), aflatoxin B_{2a} (AFB_{2a}), and AFL were obtained from prior *in vitro* incubations (Hsieh, Dalezios, Krieger, Masri & Haddon, 1974; Salhab & Hsieh, 1975). All solvents used in this study were nanograde.

Cell culture. Adult rat hepatocytes were isolated from Sprague–Dawley male rats (180–220 g) and cultured in a chemically defined culture medium, previously shown to maintain *in vivo* levels of cytochrome *P*-450 (Decad, Hsieh & Byard, 1977). Hepatocytes were cultured on collagen-coated plastic culture dishes (Pariza, Yager, Goldfarb, Gurr, Yanagi, Grossman, Becker, Barber & Potter, 1976) and incubated at 27°C in a humidified 5% CO_2–95% air incubator. After 24 hr in culture, 83 μg of [^{14}C]AFL (0·5 Ci/mol) dissolved in 0·1 ml dimethylsulphoxide was added in 20 ml of fresh culture medium to each plate containing 15×10^6 hepatocytes per plate. Control plates contained heat-killed hepatocytes (75–80°C for 45 min). Portions of culture medium (1 ml) were removed from each plate at selected time intervals, including immediately after adding fresh medium with [^{14}C]AFL and after 2, 5, and 10 hr of incubation.

Intravenous administration of [^{14}C]AFL. [^{14}C]-AFL was administered to Sprague–Dawley rats at 0·4 mg/kg. Intravenous administration of AFL allowed observation of the metabolism and toxicokinetics of AFL *in vivo* without the complication of absorption kinetics. The rats were anaesthetized with diethyl ether, secured dorsally to a thermally-regulated surgical platform, and blunt dissection revealed both jugular veins which were catheterized using a needle cannula. The needle cannula consisted of a 27-gauge needle point secured to a 10 μm capillary tube for support, which in turn was connected onto a 1 ml syringe. All joints were sealed using a cyano-acrylate adhesive (Permabond 102, Pear Chemical Co., N. Miami Beach, FL). Appropriate doses of [^{14}C]AFL were transferred in chloroform to half-dram vials, dried under nitrogen, and resuspended in a 0·9% NaCl–dimethylsulphoxide (3:1) solution. The substrate was taken up in needle catheters and administered by cannula to allow rapid infusion of the toxin directly into the jugular vein. Subsequent blood samples (0·4 ml) were collected from the opposing vein every 5 to 10 min for 1 hr. Upon administration of the prepared dose the exact administered dose was calculated as the difference between the prepared dose and the residual radioactivity detected in the dram vial, syringe and catheter by scintillation counting.

Extraction and analysis of metabolites. Portions of collected plasma or culture medium were placed in 10 ml of Handifluor® (Mallinckrodt, St. Louis, MO), for scintillation counting. All radioactivity determinations were performed in a Packard Tri-Carb, Model 2425, liquid scintillation spectrometer, equipped with automatic external standardization. [^{14}C]Toluene (New England Nuclear, Boston, MA) was used as an internal standard for quench correction. The recovered biological samples were treated with equal volumes of ice-cold methanol, and aflatoxin metabolites were extracted three times with chloroform, using centrifugation to sharpen partitioning phases. The resulting phases, which included the chloroform phase, a precipitated protein layer, and a residual aqueous phase, were used for scintillation counting. Precipitable plasma proteins required pre-treatment with Protosol® (New England Nuclear) and heating for 30–60 min at 70–80°C. Radioactivity presumably bound to cellular macromolecules in hepatocyte culture was determined after 10 hr incubation, as follows. Each plate was washed three times with 10 ml buffer described by Dulbecco & Vogt (1954). Then 10 ml of 5% trichloroacetic acid (TCA) was added to the plate. The precipitated cells were scraped from the plate with a rubber spatula, and the cells were collected on a glass fibre filter (4·25 cm GF/C, Whatman Ltd., England). The plates were each washed twice with 10 ml of 5% TCA, and the wash was also passed through the filter. The filters were then washed once with 10 ml of methanol and seven times with 10 ml of chloroform. The dried filters were placed in teflon-sealed scintillation vials with 0·5 ml Protosol® overnight at room temperature. Then, 10 ml of Patterson-Greene scintillation cocktail (Patterson & Greene, 1965) was added to each vial and radioactivity was determined.

All chloroform-extractable fractions recovered from plasma and culture medium were evaporated to dryness under nitrogen and resuspended in 500 μl of

benzene–acetonitrile (98:2). A sample of the extract (250 μl) was then applied by syringe to Quantum Linear-Q Preadsorbent silica gel thin-layer chromatoplates (250 μ, Quantum Industries, Fairfield, NJ). Authentic AFB_1 metabolites, including AFL, AFQ_1, AFLH, AFM_1, AFP_1, and AFB_{2a}, were also spotted and co-chromatographed using a series of chromatographic developing systems. The chromatoplate was first developed in diethyl ether which isolated AFL, then chloroform–acetone–water (88:12:1·5) which separated AFB_1 and its known metabolites, and finally benzene–ethanol (40:6) which separated AFM_1 and AFB_{2a}. The observed metabolites of AFB_1 were subsequently identified by their respective fluorescence under UV and corresponding R_F values compared to co-chromatographed standards. The adsorbent in each TLC spot was collected from the chromatoplates by vacuum in a 3-in Pasteur pipette plugged with glass wool, and was then transferred to scintillation vials containing 0·5 ml of distilled water and 1·0 ml of PCS™ (Amersham/Searle, Arlington Heights, IL) for hydration which aids in the release of silica-bound aflatoxin.

Quantitative TLC measurements were performed on a Schoeffel Model SD 3000 recording spectrodensitometer operated in the fluorescence mode. Identification of AFB_1 and AFL by TLC was accomplished using several TLC systems including benzene–ethanol (40:6), ethyl acetate–chloroform (3:1), diethyl ether, chloroform–acetone–isopropanol (85:15:2·5), chloroform–methanol (9:1), and diethyl ether–methanol–water–acetone (80:4·5:1·5:10).

A high-pressure liquid chromatograph (Altex Model 100, Berkeley, CA) equipped with a Dupont 836 ultraviolet and fluorescence detector was used for further confirmation and purification of AFB_1. The mobile phase used was methylene dichloride saturated with water, 0·5% methanol, and 0·1% acetic acid. The chromatograph was operated at 3160 psi, maintaining a flow rate of 1·8 ml/min. All separations were done on an Altex Lichrosorb Sil 60 (24 cm × 2·1 mm) column (Altex, Berkeley, CA).

Mutagenicity assay. The relative mutagenic potency of AFB_1 and AFL to *Salmonella typhimurium* TA98 was determined as a confirmatory assay for the biological reactivities of AFB_1 and AFL. Metabolic activation was provided by hepatic post-mitochondrial (S9) enzyme preparation derived from Charles River male white rats (200–250 g). The assay was performed according to the procedures previously described (Ames, Durston, Yamasaki & Lee, 1973) and modified (Wong & Hsieh, 1976) for use with aflatoxins.

RESULTS

Biotransformation of AFL to AFB_1 in rat hepatocyte culture

Before *in vivo* studies with [^{14}C]AFL, rat primary hepatocyte culture was used to determine whether the

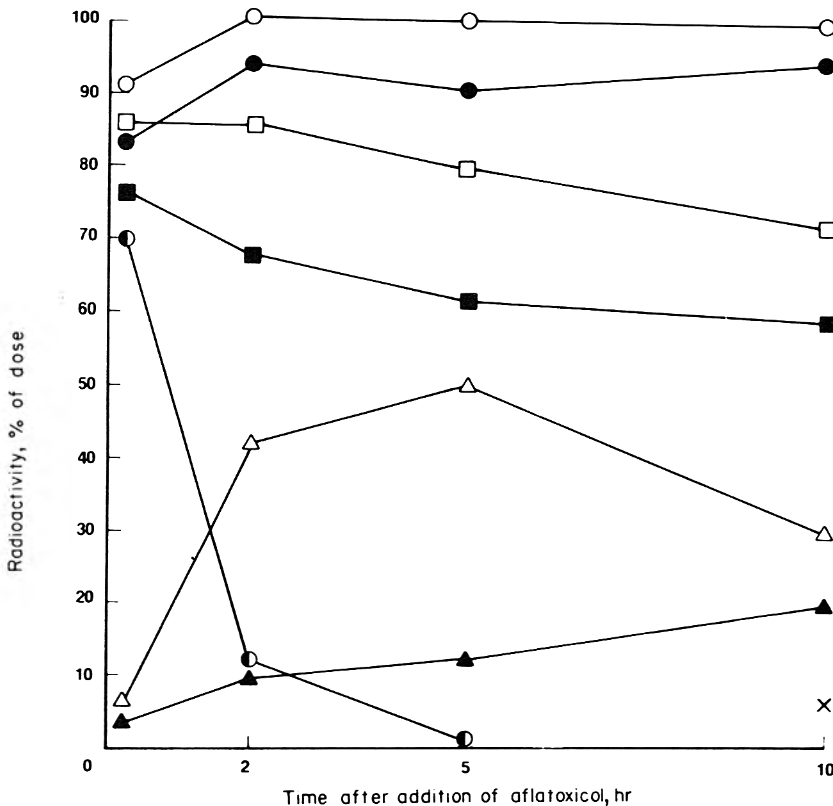

Fig. 2. Metabolism of radiolabelled aflatoxicol in primary hepatocyte culture. Radioactivity in control culture medium (heat-killed hepatocytes): total (○) and aflatoxicol levels (●). Radioactivity in cultured hepatocyte medium: total (□); chloroform extract (■); AFB_1 (△); residual aqueous (▲); aflatoxicol (◐); 5% TCA precipitated cultured hepatocytes (×).

Table 1. *Analytical evidence for the biotransformation of aflatoxicol to aflatoxin B_1 in rat plasma* in vivo *5 min after iv dosing with aflatoxicol, and in primary adult hepatocyte culture after 10-hr incubation with aflatoxicol*

Analytical technique	Results for: Metabolite from plasma (*in vivo*)	Metabolite from hepatocyte culture	Aflatoxin B_1 standard
TLC R_F using solvent system:			
Chloroform–acetone–isopropanol (85:15:2·5)	0·77	0·75	0·75
Diethyl ether	0·17	0·17	0·16
Diethyl ether–methanol–water–acetone (80:4·5:1·5:10)	0·74	0·75	0·77
Benzene–ethanol (40:6)	0·45	0·45	0·45
Chloroform–methanol (9:1)	0·90	0·93	0·91
Autoradiographic confirmation	+	+	+
HPLC R_t (min)*	5·73	—	5·61
UV spectra in methanol; λ_{max} (nm)	—	360, 257	360, 257
Increase in mutagenicity† relative to aflatoxicol (%)	—	120	120

TLC = Thin-layer chromatography HPCL = High-pressure liquid chromatography
λ_{max} = Maximum wavelength
*The mobile phase used was methylene dichloride saturated with water—0·5% methanol—0·1% acetic acid.
†Mutagenicity in the Ames Assay.

conversion of AFL to AFB_1 could be demonstrated. Thus, [^{14}C]AFL was administered to cultured rat hepatocytes at a dose level of 83 μg/15 × 10^6 cells, and metabolism was determined over a 10-hr time-course (Fig. 2). There was a rapid biotransformation of AFL to AFB_1 as well as the formation of several other chloroform-soluble AFB_1 metabolites, including AFM_1. The total radioactivity and chloroform-extractable activity declined slowly. After 2 hr, the levels of AFL declined to only 10% of the administered dose, while AFB_1 levels rose to over 40% of the dose. AFB_1 reached a peak level at 5 hr, representing about 50% of the dose, while AFL at this time had declined to a negligible level. While AFL was not detected after 5 hr, AFB_1 levels remained elevated. Water-soluble metabolites were also formed and in-

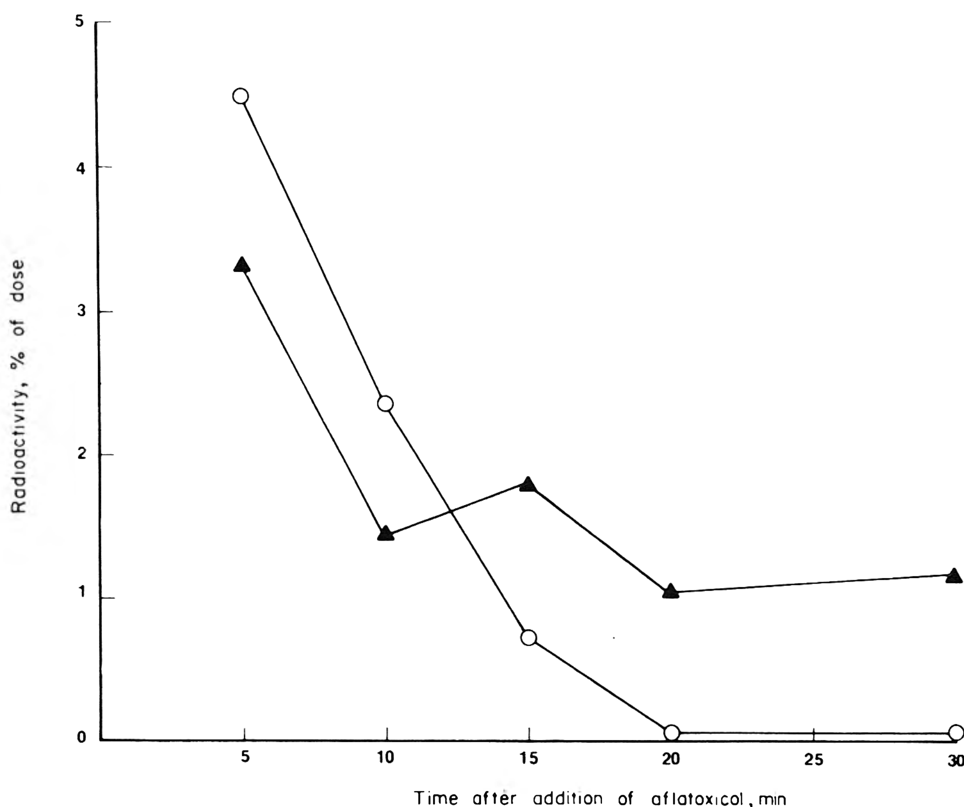

Fig. 3. The bioconversion of AFL to AFB_1 in Sprague–Dawley rats. [^{14}C]AFL was dosed iv at a level of 0·4 mg/kg. Concentrations of AFB_1 (▲) and AFL (○) in blood sample extracts were determined by scintillation counting and values were calculated assuming a total plasma volume of 40·4 ml/kg body weight. Points plotted are mean values derived from two rats.

creased steadily during the incubation. The radioactivity bound to the hepatocytes that was recovered after the 10 hr incubation was approximately 6% of the total dose. The AFB_1 isolated from the medium of hepatocyte cultures incubated with AFL was identified by thin-layer chromatography, autoradiography, ultraviolet absorption spectroscopy, and by its increased mutagenicity to *Salmonella typhimurium* TA98 (Table 1).

Biotransformation of AFL to AFB_1 in vivo

On administration of $[^{14}C]$AFL in the rat, there was rapid clearance of AFL from plasma, exhibiting a relatively high volume of distribution (Fig. 3). Observing the plasma time-concentration profile, there was an apparent active bioconversion of AFL to AFB_1. At 5 min after dosing, the total plasma levels of AFB_1 and AFL were nearly equal. After 20 min, most of the observed plasma radioactivity was identified as $[^{14}C]AFB_1$, while $[^{14}C]$AFL levels had declined to negligible values. From data obtained previously (Wong & Hsieh, 1978), the plasma biological half-life of AFB_1 in the rat was calculated to be approximately 28 min. Using the same calculation procedure, the biological half-life for AFL was estimated to be 7 min. The $[^{14}C]AFB_1$ recovered from the *in vivo* conversion of $[^{14}C]$AFL was identified by thin-layer and high pressure liquid chromatographic analyses and by autoradiography (Table 1). The quantity of AFB_1 obtained from plasma was too small to allow confirmation by spectroscopic analysis and mutagenicity assay.

DISCUSSION

Our recent comparative metabolism and toxicokinetic studies in the rat, mouse, and monkey *in vivo* have revealed that the rat characteristically produced AFL *in vivo*, while the monkey and mouse apparently metabolized AFB_1 mainly to AFM_1 and AFP_1 (Wong, Wei & Hsieh, 1978). The identification of AFL as the major characteristic plasma metabolite of AFB_1 in only the highly susceptible rat (Wong & Hsieh, 1978) has greatly enhanced the toxicological significance of this metabolite, since its formation has been correlated with an animal's sensitivity to the acute and carcinogenic effects of AFB_1 (Patterson, 1973; Edwards, Rintel & Parker, 1975; Hsieh *et al.* 1977; Wong & Hsieh, 1978).

So far, the most attractive proposal regarding the role of AFL in the aetiology of carcinogenic aflatoxicosis is that AFL can be enzymatically converted in target hepatocytes to the parent compound, AFB_1, followed by bioactivation of the latter to produce toxicity. Previous studies have demonstrated that AFL can be biotransformed back to AFB_1 *in vitro* using liver preparations isolated from a variety of animal species (Loveland *et al.* 1977; Salhab & Edwards, 1977a) including the rat. The results of the present study provide evidence that AFL can be actively biotransformed to AFB_1 in the rat *in vivo* and in primary hepatocyte cultures.

AFL appears to be highly lipophilic as shown by its mobility in the TLC system developed with diethyl ether (Patterson & Roberts, 1971; Hsieh *et al.* 1977) and also by its apparent high level of distribution to the tissues (Fig. 3). The extent of tissue penetration due to lipid solubility is probably responsible for the observed rapid bioconversion of AFL to AFB_1, which was found to occur in liver cells (Salhab & Edwards, 1977a). The shorter plasma biological half-life of AFL (7 min) compared with that of AFB_1 (28 min), is probably a result of active metabolic conversion of AFL to AFB_1 and other metabolites following the rapid penetration of AFL. The lipophilic nature of AFL presumably also facilitates its intracellular migration to target sites.

As suggested by Neal & Godoy (1976), the crucial bioactivation of AFB_1 may occur at the target nuclear membrane since the presumed ultimate carcinogen, the AFB_1-epoxide, is considered too reactive and unstable for microsomal mediation to DNA binding. Also no protein carrier systems have been found to suggest a cellular transport of the epoxide. Therefore, it is possible that the "reservoir" of AFB_1 created by the reversible formation of the lipophilic AFL from AFB_1 may increase the intracellular bioavailability of AFB_1 for nuclear activation through facilitated intracellular migration of AFL.

Rat hepatocyte culture serves as a valuable adjunct to *in vivo* studies of AFB_1 metabolism, since this "*in vitro* system" provides the best model of *in vivo* hepatocyte activity (Decad, Hsieh & Byard, 1977). Some of the additional advantages this technique offered in this study included the opportunity to study hepatic metabolism alone without the complication of extra-hepatic binding and metabolism of aflatoxin as seen *in vivo*, a much smaller dose of $[^{14}C]$AF required for adequate sensitivity, and a higher total recovery of AFL and its metabolites produced in cell culture to aid in subsequent identification and characterization studies.

In a previous study, hepatocyte cultures with *in vivo* levels of cytochrome P-450 metabolized AFB_1 to primarily water-soluble metabolites, covalently-bound species, and AFM_1 (Decad *et al.* 1977) which is invariably the major identifiable urinary metabolite of AFB_1 *in vivo* (Campbell, Caedo, Bulatao-Jayme, Salamat & Engel, 1970; Dalezios, Hsieh & Wogan, 1973; Wong *et al.* 1978). AFL, however, was not detected as an AFB_1 metabolite in the cell culture, which represents an apparent discrepancy between the *in vivo* condition and the hepatocyte culture. The biochemical composition of the hepatocyte culture medium probably has a significant influence on the production and recovery of AFL and is presently under investigation. Since AFL is shown to be highly lipophilic, it is possible that AFL is more readily partitioned into the highly lipoidal blood and therefore recovered more readily *in vivo*. It is interesting to note that the hepatocyte-bound activity at 10 hr after AFL administration represented only 6%, whereas for AFB_1, about 12% of the dose was found unextractable (Decad *et al.* 1977). This finding is consistent with the lower mutagenic potency of AFL as compared to that of AFB_1 and that the bioactivation of AFL is probably through its reversion to AFB_1 rather than the other way around.

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EXPOSURE TO N-NITROSODIMETHYLAMINE IN A LEATHER TANNERY

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Abstract—*N*-Nitrosodimethylamine (NDMA) was detected in the atmosphere of a leather tannery at levels of up to 47 $\mu g/m^3$. As far as we know this is the highest occupational exposure to this potent animal carcinogen yet discovered. NDMA was detected in all the air samples taken at different sites in the tannery on three visits in 1978. The highest level was in the re-tanning, colouring and fat-liquoring area. The average atmospheric level of NDMA on the first and second visits was 19 $\mu g/m^3$. The levels were considerably reduced on the third visit, when the tannery had been thoroughly cleaned, and ranged from 0·1 to 3·4 $\mu g/m^3$. Small amounts of NDMA were also found in process and waste water. The tannery examined uses basic processes that are standard within the industry; therefore, NDMA may also contaminate the atmosphere of other tanneries. Exposure of tannery workers to NDMA suggests an area for epidemiological studies, as well as for further analytical work, which could contribute to a knowledge of the possible carcinogenic effects of NDMA in man.

INTRODUCTION

Since 1956 when Magee and Barnes demonstrated the carcinogenic effects of *N*-nitrosodimethylamine (NDMA) in the rat, NDMA has become the most extensively studied member of this class of carcinogens. It has been shown to cause cancer in a variety of organs in virtually every species in which it has been tested (Magee, Montesano & Preussmann, 1976). A recent study by the International Agency for Research on Cancer (1978) concludes that: "the general (human) population may be exposed to low levels of *N*-nitrosodimethylamine; however, no exposed group suitable for an epidemiological investigation has yet been identified". We report here on the possibility that leather tannery workers may constitute just such a group. We are unaware of any epidemiological studies that have specifically focused on such workers, although there have been studies on bootmakers (Acheson, 1976) and "leather operatives" (Viadana, Bross & Houten, 1976).

As part of our study of human exposure to *N*-nitrosamines, we have been examining work environments in which amines are used. Leather tanning was chosen because dimethylamine sulphate is used as a depilatory agent in some tanneries (Walker, Gordon, Thomas & Ouellette, 1976). For the initial study we chose a tannery in New England employing about 300 workers and processing about 2000 hides per day in which the method of tanning used was characteristic of the industry (New England Tanner Club, 1977).

EXPERIMENTAL

Tannery visits. The selected tannery was in a one-storey building, divided into wet- and dry-operations areas. Some sites were used for several processes; for instance, at the re-tanning site, colouring, dyeing and fat-liquoring were also carried out. Three visits were made to the factory and the entire leather tanning and finishing processes were studied. On the first visit (11 April 1978) six air samples representing a cross section of the air in the tannery were collected and on the following visit (13 April 1978) 20 air samples were taken. In addition, on the second visit, numerous bulk samples were taken. The third visit was made on 1 June 1978 when 10 air samples and numerous bulk samples were collected. A total of 27 bulk samples were examined for *N*-nitroso compounds and these consisted of 11 of chemicals or chemical mixes, two of hide and leather, two of waste water, and 12 of process water from the wet-operations area of the tannery.

Apparatus. Laboratory equipment, including gas- and high-pressure liquid chromatographs (GC, HPLC), thermal energy analyser (TEA), fume hood and detectors, was located inside a fully equipped mobile laboratory (Krull, Fan, Fine, Young & Egan, 1978), which was parked near the tannery. An HPLC–TEA was constructed (Fine, Rounbehler, Pellizzari, Bunch, Berkley, McCrae, Bursey, Sawicki, Krost & DeMarrais, 1976) by combining a high-pressure pump (Altex model 110, Altex Corp., Berkeley, CA) with an injector (Rheodyne model 7120, Altex Corp.), a stain-

less steel column (4 × 39 mm) packed with 10 μ Lichrosorb Si60 (E. Merck Labs, Elmsford, NY) and a TEA detector (TEA model 502LC, Thermo Electron Corp., Waltham, MA). For GC–TEA, a single column isothermal GC (Thermo Electron Corp.) was constructed from 14 ft of stainless steel tubing (outer diameter 1/8 in.) packed with 10% Carbowax 20M containing 0·5% KOH on Chromosorb W HP (80–100 mesh) interfaced to a TEA (Fine & Rounbehler, 1975). The column temperature was 160°C, and the carrier gas was argon (flow-rate 15 ml/min).

The GC–flame ionization detector (FID) used was a Hewlett-Packard 5710 (Palo Alto, CA) equipped with the same GC column used for GC–TEA.

Chemicals. Dilute standard solutions of NDMA and *N*-nitrosomorpholine (NMOR) were obtained from the Analytical Services Laboratory of Thermo Electron Corp. Dichloromethane, isooctane, acetone and diethyl ether, of a grade distilled in glass, were obtained from Burdick and Jackson (Muskegon, NJ). AnalaR grade potassium hydroxide (KOH), sodium sulphate, sulphuric acid and dimethylamine hydrochloride were used.

Collection and analysis. Air samples were collected (Fine, Rounbehler, Sawicki & Krost, 1977) in 1 N-KOH traps by passing air at a flow rate of between 1 and 2 litres/min through a glass impinger containing 45 ml of 1 N-KOH. For analysis, the KOH solutions were extracted with dichloromethane and concentrated at 52°C in a Kuderna-Danish evaporator (Kontes, Vineland, NJ) to a volume of 0·5–1·0 ml. All of the concentrates were analysed by GC–TEA and/or HPLC–TEA (Fine, Rounbehler, Silvergleid & Ross, 1977). For NDMA and NMOR, the HPLC–TEA was operated isocratically using 2 ml/min of *n*-hexane–acetone (85:15, v/v). HPLC–TEA screening for other *N*-nitrosamines (Fan, Krull, Ross, Wolf & Fine, 1978) was carried out using the following solvent systems: acetone–isoctane in the ratios 50:50, 25:75 and 85:15 v/v.

All quantitative data on atmospheric NDMA were obtained from air samples collected in 1 N-KOH traps. In order to demonstrate that NDMA was not being formed in the traps, three other trapping techniques were also used. Two air samples were collected on solid dry Tenax GC material (obtained from Applied Science, State College, PA; Fine *et al.* 1976). The 10 × 1·5 cm cartridges were eluted with 75 ml of ether, dried over sodium sulphate, filtered and concentrated to 0·5 ml on a Kuderna-Danish evaporator. Two samples each were also collected on a PREPTUBE (model 117 Thermo Electron Corp.) and on a cellulose based material (Tampax tampon, Tampax, Inc., Palmer, MA) both of which had been soaked in 1 N-KOH. All four cartridges were eluted with dichloromethane and the eluate concentrated on a Kuderna-Danish evaporator. The three alternative techniques, after correcting for low efficiencies and recoveries, gave data that were indistinguishable from those obtained with the KOH traps.

For mass-spectral analysis of NDMA in the air samples, the contents of several traps were combined, concentrated and quantitated by HPLC–TEA, GC–TEA and GC–FID. The structures of these chromatographically-purified TEA-positive fractions were confirmed by one of two mass-spectral methods, depending on the amount of sample available. For microgram quantities of sample, low resolution gas-liquid chromatography–mass spectrometry (GC–MS) was used and the entire mass range of each eluting GC peak was magnetically scanned at 4 sec intervals using a Hitachi RMU6 instrument (Perkin-Elmer, Norwalk, CT). The combination of GC retention time and mass spectral identity was used to confirm structure. Nanogram quantities of sample were analysed by high resolution GC–MS (Gough, Webb, Pringeur & Wood, 1977) using a Varian MAT Ch-7 mass spectrometer (Bremen, Federal Republic of Germany). The magnetic analyser was set to resolve the exact mass of NDMA ($C_2H_6N_2O$–74·0480; resolution ~9000, peak-matched against a perfluoroalkane standard), from other background materials. Repetitive scanning over the *m/e* 74 nominal mass region resolved four minor components only one of which increased coincident with GC elution time of NDMA. Sample analysis and the detection of an exact mass fitting the composition for the molecular-ion of NDMA combined with coincident GC retention time, were used to confirm the presence of NDMA.

For the solid and chemical samples, 1 g was dissolved or soaked (for 30 min) in 5 ml of acetone and then filtered through approximately 2 g of sodium sulphate. The filtrate was analysed by GC–TEA and HPLC–TEA. For the water samples, 15 ml was loaded onto a PREPTUBE which had been presoaked with 15 ml of dichloromethane. The PREPTUBE was eluted with 30 ml (3 × 10 ml) dichloromethane, and the eluate concentrated in a Kuderna-Danish evaporator. The concentrate was analysed by GC–TEA and HPLC–TEA. The detection limit was 0·1 μg/g for solid and chemical samples and 0·001 μg/ml for the water samples.

RESULTS AND DISCUSSION

On the first visit to the tannery NDMA was found in all six air samples at levels ranging from 6 μg/m³ in the spray finishing area to 33 μg/m³ near the chrome-tanning operation. All the air samples taken inside the tannery on the second visit contained NDMA, including those collected in the lunch room (0·1 μg/m³) and in the shipping room (1·4 μg/m³), although the levels in the grading and staking areas were not significant. The combined data from both visits are shown in Fig. 1. The highest NDMA level of 47 μg/m³ was found in the re-tanning, colouring and fat-liquoring area. The average amount of airborne NDMA found on the two visits was 19 μg/m³. On both the first and second visits, the floor was partially covered with decaying animal flesh and fat. The odour of putrified flesh was overpowering to the uninitiated. In addition, parts of the floor were wet and slippery with process water. Because of the cool weather, ventilation was by only a few fans mounted near the ceiling.

In addition to NDMA, NMOR at a level of 2·0 μg/m³ was found in three air samples taken in the chemical doping area on the second visit. Two of the samples at this location were collected using a Tenax GC dry trap (Fine *et al.* 1976). The identity of the NMOR was confirmed by its retention time on both GC–TEA and HPLC–TEA.

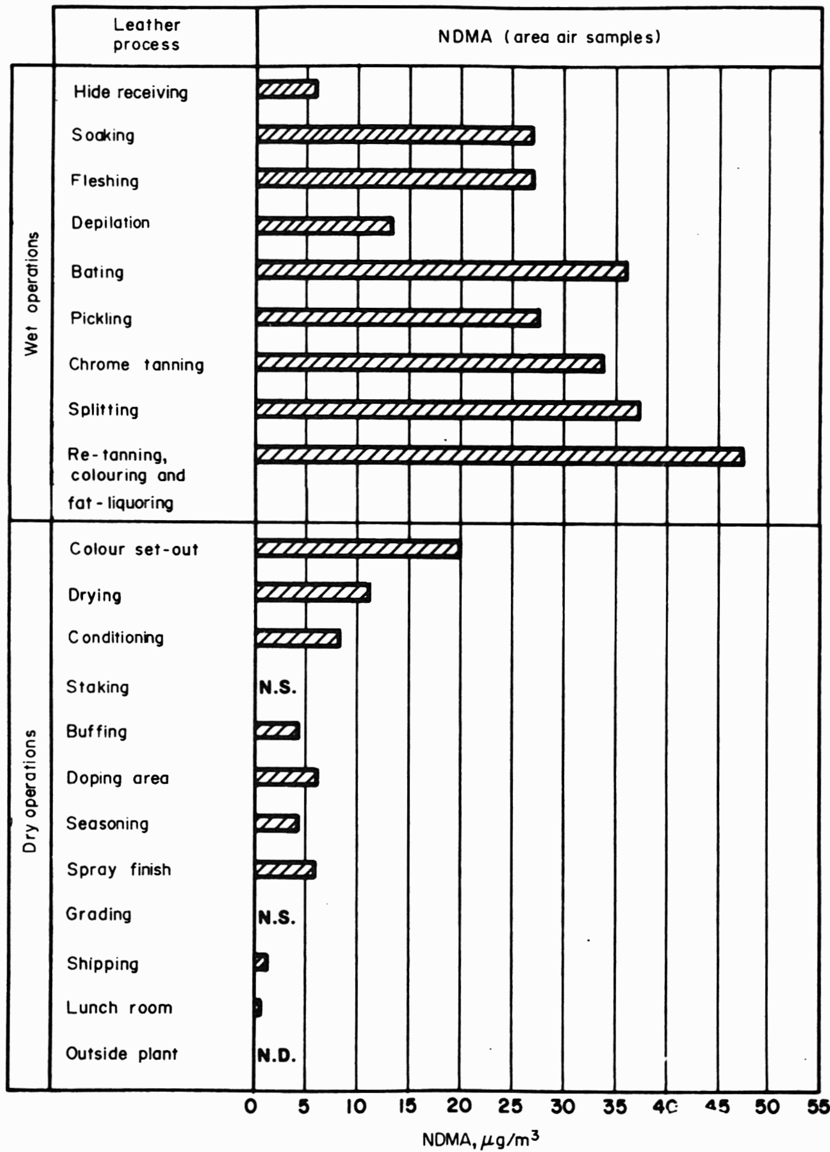

Fig. 1. The highest levels of NDMA in the atmosphere at various stages of the tanning process (combined data from the first two visits): ND = not detected; NS = not significant.

By the third visit the tannery had been thoroughly cleaned. The floors were clean and dry, there was no sign of decaying flesh or fat and no odour of putrified flesh. In addition, the hot weather necessitated the opening of all the ventilation windows and doors. On this visit NDMA was again found in the atmosphere at all sites, but the levels were considerably reduced, ranging from 1·1 to 3·4 $\mu g/m^3$.

Only four of the 27 bulk samples contained NDMA (Table 1). The highest level of NDMA (0·5 $\mu g/g$) was found in a sample from a 36·5% aqueous solution of dimethylamine sulphate. According to plant personnel, 117 litres of dimethylamine sulphate are used each day in the depilation process. This would amount to less than 60 mg NDMA/day from the precontaminated amine. NDMA (0·0014 $\mu g/ml$) was also found in the process water of the re-lime pit. This process water contained 5·7 litres of dimethylamine sulphate in 8000 kg of lime-saturated water and is used to remove hair from the raw hides. The other samples which contained NDMA (0·004 $\mu g/ml$ and 0·006 $\mu g/ml$) were waste-water samples from the tannery outlet pipe collected at the local municipal waste-water treatment plant. We were informed that the amount of waste water entering the waste-water treatment plant from the tannery was about $3{\cdot}8 \times 10^6$ litres/day. Approximately 20 g of NDMA would be required to produce 0·006 $\mu g/ml$ in this amount of water (assuming no losses by evaporation, breakdown, etc.). A sample of the salted hide was also examined for NDMA, but none was detected (detection limit 0·5 $\mu g/g$). This salted cowhide was further examined by placing a 1 g piece in 5 ml of sulphuric acid (pH 3) containing 50 mg of dimethylamine hydrochloride for 4 hr at 25°C. This test was carried out to determine if the raw cowhide itself contained any material which could possibly nitrosate dimethylamine, even under ideal conditions. At a detection

Table 1. *NDMA levels in liquid and solid bulk samples**

Sample description	NDMA (μg/g)
Chemicals	
Azo Rubine Dye	ND
Penetrator L-219	ND
Nigrosine Blue L	ND
Polar Sol 5	ND
Betz Formula NA-6	ND
Boiler rust inhibitor mix	ND
Ammonia paste wash	ND
P-Nitrophenol	ND
KITO-40 (fungicide)	ND
Fresh brine	ND
Aqueous dimethylamine sulphate (36·5%)	0·5
Leather samples	
Chrome-tanned leather	ND
Raw-salted cowhide	ND
Waste water	
Beam-house waste water	0·004
Tanning-house waste water	0·006
Process water	
Re-lime pit	0·0014
Bating solution (two samples)	ND
Pickling solution (two samples)	ND
Chrome-tanning solution (two samples)	ND
Final rinse from chrome tanning (two samples)	ND
Wash out of colouring	ND
First rinse from fat-liquoring	ND
Final rinse from fat-liquoring	ND

ND = Not detected
*Combined data from visits 2 and 3.

limit of 0·04 μg/g neither NDMA, nor any other TEA-responsive compounds (*N*-, *C*- and *S*-nitro and nitroso) were found.

The NDMA impurity in the dimethylamine sulphate used in the depilation step is insufficient to account for either the atmospheric or aqueous NDMA levels. If the dimethylamine moiety were somehow nitrosated in the tannery, then sufficient dimethylamine sulphate is used daily to account for the observed NDMA. However, a possible nitrosating agent has not been identified. Analysis of the various process solutions used in the wet area where the NDMA levels are highest did not reveal the source of the NDMA. We do not know how the NDMA in the tannery is being produced, and are unable to speculate as to a possible mechanism of production. Much further work is needed to determine the source of the NDMA in the tannery.

Although atmospheric NDMA was not found outside the original tannery, the most recent field studies, carried out in August 1978 did show NDMA to be present in the air immediately adjacent to tanneries in New Hampshire (0·10 μg/m^3) and in New Jersey (0·15 μg/m^3). It was concluded from these very limited studies that NDMA is probably present in and near most tanning facilities.

The significance of these findings may be inferred from the results of a recent study by Moiseev & Benemansky (1975). They reported that 30 male Wistar rats breathing air containing 220 μg NDMA/m^3 continuously for 25 months showed an increase in malignant tumours from 13% in the controls to at least 83% in the treated animals. These tumours were mainly of the liver and kidney. In the same experiment, female rats were exposed for 25 months and Balb/C mice (both male and female) for 17 months. These groups showed significant increases in benign tumours, especially of the lung and kidney. We estimate that the male rats in this study were exposed to NDMA at 525 μg/m^2 of body surface area/day (the rats were assumed to have an average weight over the experimental period of 400 g, a body surface area of 0·054 m^2 and to breathe 0·09 litres/min). This represents a total exposure to 400 mg/m^2 (54 mg/kg) over the course of the experiment, an NDMA dose similar to those reported by other laboratories to cause tumours in several species when administered both by the oral and inhalation routes (Clapp & Toya, 1970; Druckrey, Preussman, Ivankovic & Schmahl, 1967; Terracini, Magee & Barnes, 1967; Tomatis & Cefis, 1967).

Assuming that an average worker weighs 70 kg (1·85 m^2 body surface area) and breathes 20 litre/min for an 8-hr working day, then a worker breathing airborne NDMA at 47 μg/m^3 in the wet process area, will be exposed to NDMA at a level of 240 μg/m^2 of body surface area/day. Thus the highest exposure level found corresponds to only slightly less than half the exposure which caused an increase in the incidence of malignant tumours in male rats from 13 to 83%. Even in the shipping room the exposure is 7 μg/m^2/day or approximately 1% of the dose used in the rat study.

As far as we know, this is the highest level of

chronic exposure to NDMA yet reported. Since certain categories of skilled workers (e.g., master tanners and hide splitters) have usually been employed in tanneries, especially the wet process area, for many years, we believe that a strong case can be made for further monitoring in other tanneries and for the initiation of corrective measures to reduce worker exposure. It also seems likely that these workers may be the first population in which the potential hazards of NDMA in humans can be assessed.

Acknowledgement—This work was supported by the following US contracts and grants: National Institute of Occupational Safety and Health (Contract 210-77-0100), Environmental Protection Agency (Contract 68-02-2766) and National Science Foundation (Grant no. ENV75-20802-AO2).

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CARCINOGENICITY OF THE HAIR-DYE COMPONENT 2-NITRO-*p*-PHENYLENEDIAMINE: INDUCTION OF EOSINOPHILIC HEPATOCELLULAR NEOPLASMS IN FEMALE B6C3F1 MICE

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Abstract—Groups of 50 male and 50 female B6C3F1 mice were given 2-nitro-*p*-phenylenediamine in the feed at either 2200 or 4400 ppm for 78 wk. Twenty animals of each sex were used as controls. Treatment was followed by a 12–13 wk observation period. There was a dose-related depression of body-weight gain in both sexes. Pigmentation was observed in liver, spleen, intestine and thyroid. Female mice receiving 2-nitro-*p*-phenylenediamine showed a dose-related increase in the incidence of hepatocellular neoplasms. The majority of these neoplasms were multiple eosinophilic hepatocellular adenomas which were different from the adenomas in control mice. A few female mice given 2-nitro-*p*-phenylenediamine had foci of cellular alteration in the liver. Neoplasms similar to those induced by 2-nitro-*p*-phenylenediamine have been reported in mice exposed to two other aromatic amines, 4-chloro-*m*-phenylenediamine and 4-nitro-*o*-phenylenediamine. However the latter did not prove to be carcinogenic.

INTRODUCTION

2-Nitro-*p*-phenylenediamine (2-NPPD) is used in hair and in fur dyeing to produce a red-brown colour or to add red shading when combined with other oxidation bases (Society of Dyers and Colourists, 1956). It is able to penetrate hair shafts and is one of the most commonly used dyes in semipermanent and permanent hair-dye preparations (Burnett, Goldenthal, Harris, Wazeter, Strausburg, Kapp & Voelker, 1976; Corbett & Menkart, 1973; Markland, 1966). It is estimated that up to 40% of American women may be regular users of hair dyes (Corbett & Menkart, 1973; Marzulli, Green & Maibach, 1978).

It was reported that 2-NPPD had no teratogenic effect in rats (Burnett *et al.* 1976; Wernick, Lanman & Fraux, 1975) or in rabbits (Wernick *et al.* 1975). However it was mutagenic in the TA1538 strain of *Salmonella typhimurium* (Ames, Kammer & Yamasaki, 1975; Ammenheuser & Warren, 1979; Searle, Harnden, Venitt & Gyde, 1975), weakly mutagenic in a forward mutational assay system (Palmer, Denunzio & Green, 1977), and induced unscheduled DNA synthesis in HeLa cells (Martin, McDermid & Garner, 1978). Chromosomal aberrations produced by 2-NPPD have been demonstrated in lymphocytes (Searle *et al.* 1975) and in CHMP/E cells of Chinese hamster prostate gland (Kirkland & Venitt, 1976). Wynder, Onderdonk & Mantel (1963) and Anthony & Thomas (1970) reported an increased incidence of bladder cancer in dye workers. In view of this and the widespread exposure of the population to hair dyes and the lack of chronic toxicity data, the National Cancer Institute sponsored a 2-yr study in B6C3F1 mice. This report gives further details of the histopathological findings, particularly in the livers of these mice.

EXPERIMENTAL

B6C3F1 mice weighing *c.* 20 g were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA and were about 6 wk old when treatment began. They were kept under normal laboratory conditions (National Cancer Institute, 1979a) and were fed Wayne Lab-Blox meal (Allied Mills, Inc., Chicago, IL). The 2-NPPD (Ashland Chemical Co., Columbus, OH) was administered in the feed to groups of 50 female and 50 male mice at doses of 2200 or 4400 ppm. Test diet was given for 78 wk followed by a 12- to 13-wk observation period. Twenty mice of each sex were used as controls. An autopsy was performed on each animal regardless of whether it died, was killed when moribund, or was killed at the end of the study. The tissues were fixed in buffered 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Step sections were made of the livers from all mice with liver tumours. These were stained with the Gomori-stain for iron, by the acid fast (Kinyoun) method and also by the periodic acid Schiff method (PAS). Data from the experiment were recorded in a computerized data-processing system, the Carcinogenesis Bioassay Data System (Linhart, Cooper, Martin, Page & Peters, 1974). These data were analysed using the statistical techniques described in the National Cancer Institute report (1979a).

RESULTS

A dose-related depression of mean body weight was apparent in both male and female mice throughout the test period. Treated mice weighed 15–20% less than controls throughout the course of the experiment. Survival was not dose-related and 80–90% of the mice were alive after 90 wk.

Table 1. *Hepatocellular lesions in B6C3F1 mice fed diets containing 2-nitro-p-diphenylenediamine*

Dietary level of 2-NPPD (ppm)	No. of mice	No. of mice with .... Foci of cellular alteration: eosinophilic	Foci of cellular alteration: basophilic	Hepatocellular adenomas: eosinophilic	Hepatocellular adenomas: basophilic	Hepatocellular carcinomas: eosinophilic	Hepatocellular carcinomas: basophilic
			Male				
0	20	0	0	0	1	1	1
2200	50	0	0	3	1	0	1
4400	50	0	0	2	0	0	1
			Female				
0	20	0	0	0	1	0	0
2200	49	3	0	12	0	0	1
4400	49	5(2)*	0	18(7)	0	3	0

*Figures in parentheses indicate the number of mice with multiple lesions.

The occurrence of hepatocellular lesions (foci of cellular alteration, adenomas and carcinomas) was dose-related in females but not in males (Table 1).

The liver tumours that were found in the control and treated males were of the usual types and occurred at a rate which was normal for B6C3F1 mice of this age (Frith & Ward, 1979; Ward, Bernal, Buratto, Goodman, Strandberg & Schueler, 1979; Ward & Vlahakis, 1978). They were adenomas composed of small basophilic hepatocytes and carcinomas composed of basophilic hepatocytes forming prominent trabecular patterns. The hepatocellular adenoma in the control female was composed of smaller than normal hepatocytes that had basophilic cytoplasm and small round nuclei with marginal condensation of chromatin (Fig. 1).

However the adenomas in the treated female mice were very different from those in the controls. They were composed of large eosinophilic hepatocytes and were well-differentiated neoplasms in which the tumour cells were arranged in plates, one or two cells thick similar to those of the normal liver cords. The intervening sinusoids were narrowed or obstructed by expanding large neoplastic cells. Nuclear polymorphism was prominent and an occasional mitotic figure was seen (Fig. 2). In several nuclei, there were prominent pseudo-inclusion bodies (nuclear membrane invaginations) which were not acid fast (Fig. 3). The solitary adenomas consisted of cells with a granular, eosinophilic cytoplasm. Such cells were often vacuolated; a few appeared atypical and others had binucleate forms (Fig. 4). Glycogen was present in the cells of the adenomas. Seven of 18 adenoma-bearing female mice in the higher dose group had multiple hepatocellular adenomas. The carcinomas were mostly composed of eosinophilic cells forming prominent trabecular patterns. No carcinomas metastasized to distant organs. In addition to the hepatocellular adenomas and carcinomas, foci of cellular alteration of an eosinophilic type were seen in three mice without tumours of the low-dose and in five mice without tumours of the high-dose group. In the higher dose group there were two animals with multiple eosinophilic foci. All the foci contained cells with large nuclei and considerable amounts of eosinophilic cytoplasm. No control females had these foci.

The liver and other organs (spleen, thyroid, intestine) had accumulations of golden-brown pigment which did not stain by any of the three methods used. In the liver, this golden-brown granular pigment occurred mainly around periportal vessels (Fig. 5), in Kupffer cells, and around bile ducts and formed extensive, dark-brown plugs. Although it was seen only in dosed animals, it seemed to be inert and no lesions were attributed to its presence. A variety of other non-neoplastic lesions were present in both control and dosed animals (National Cancer Institute, 1979a), but they were considered to represent spontaneous alterations in this species.

The combined incidences of hepatocellular carcinomas or hepatocellular adenomas in both groups of treated female mice were shown to be significantly ($P < 0{\cdot}005$) greater than in the controls using the Cochran–Armitage test. Using the Fisher exact test, the incidence in the higher dose group was significantly ($P < 0{\cdot}007$) greater than in the controls.

DISCUSSION

Both 2-NPPD and 4-nitro-*o*-phenylenediamine (4-NOPD), also a constituent of permanent hair dyes (Fig. 6), were active when tested in a system to induce 'unscheduled DNA synthesis' in HeLa cells (Martin *et al.* 1978), although 2-NPPD was more potent than 4-NOPD. However, in mutagenicity tests in bacteria, 4-NOPD was more active than 2-NPPD (Searle *et al.* 1975; Ames *et al.* 1975). Both chemicals were either weakly mutagenic (Palmer *et al.* 1977) or non-mutagenic.

Similar feeding studies to the one described above for 2-NPPD have been carried out using 4-NOPD (National Cancer Institute, 1979b) or 4-chloro-*m*-phenylenediamine (4-CMPD; Fig. 6) an intermediate in the preparation of dyes (National Cancer Institute, 1978). Groups of male and female mice were given the compounds at low (4-NOPD, 3750 ppm; 4-CMPD, 0·5% in diet) or high (4-NOPD, 7500 ppm, 4-CMPD, 1% in diet) dose levels. The duration of treatment with 4-NOPD was 102 wk as opposed to 78 wk with 4-CMPD and 2-NPPD. Dose-related decreases in mean weight-gain occurred in both sexes with all three compounds. There was an increase in eosinophilic hepatocellular adenomas in females given the lower dose of 4-NOPD, but statistical tests showed

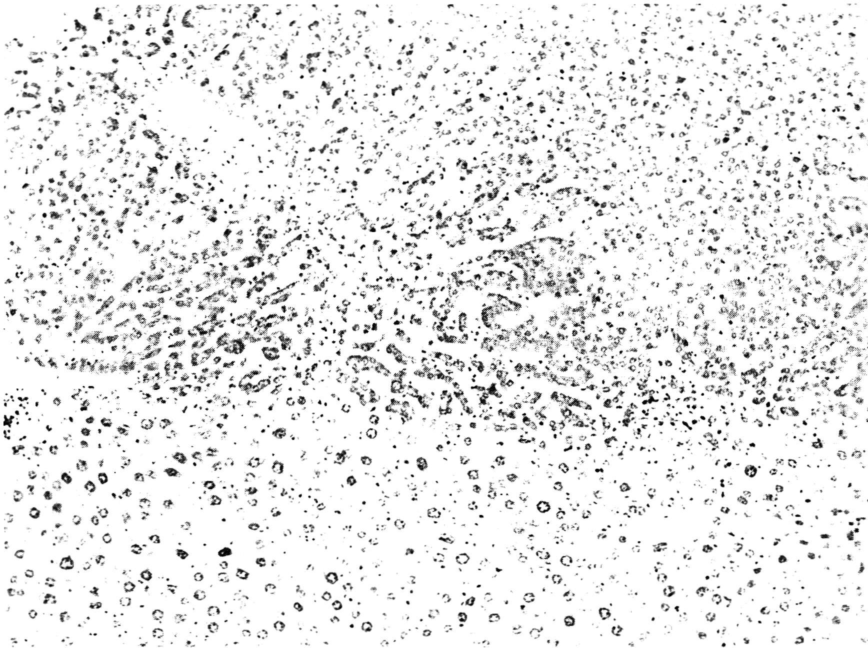

Fig. 1. Basophilic hepatocellular adenoma of a female control mouse showing a clear border between the darker stained tumour cells and normal liver tissue. The cell nuclei of the liver neoplasm are small within dark stained (basophilic) cytoplasm. Haematoxylin and eosin × 130.

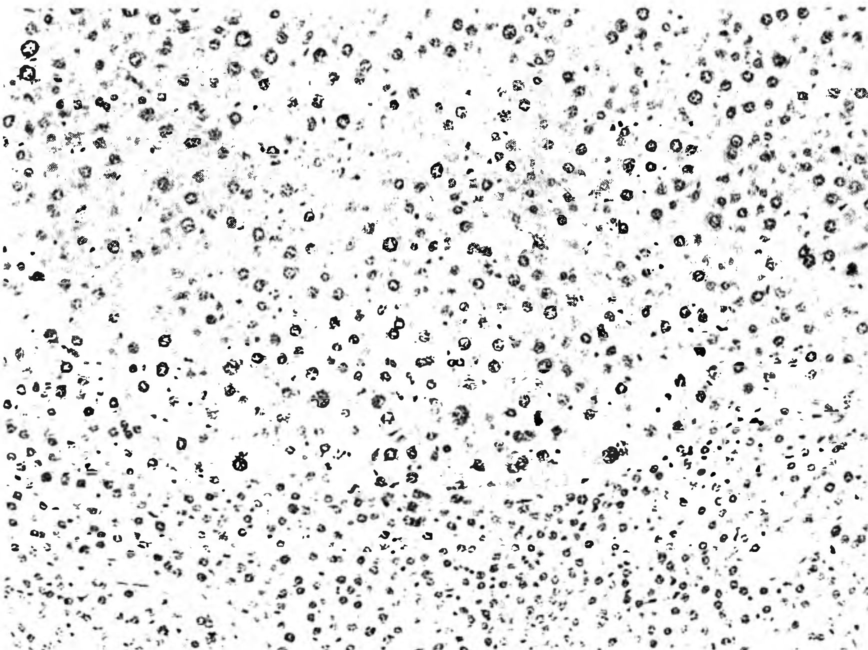

Fig. 2. Eosinophilic hepatocellular adenoma (top) adjacent to normal liver (bottom) of a female mouse treated with 2200 ppm 2-nitro-*p*-phenylenediamine for 78 wk. The hepatocytes are large with polymorphic nuclei, some mitoses and pale staining (eosinophilic) cytoplasm. Haematoxylin and eosin × 130.

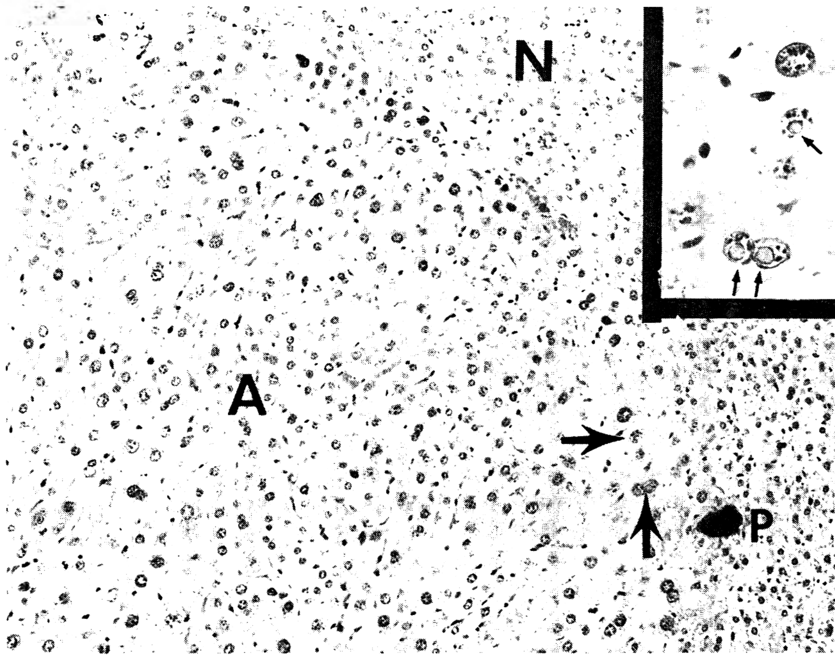

Fig. 3. Eosinophilic hepatocellular adenoma (A) next to normal liver (N) of a female mouse treated with 4400 ppm 2-nitro-*p*-phenylenediamine for 78 wk. Several nuclei (arrowed) have nuclear membrane invaginations. These are shown at greater magnification in the inset. Also pigment (P) is seen. Haematoxylin and eosin ×130 Inset: ×330.

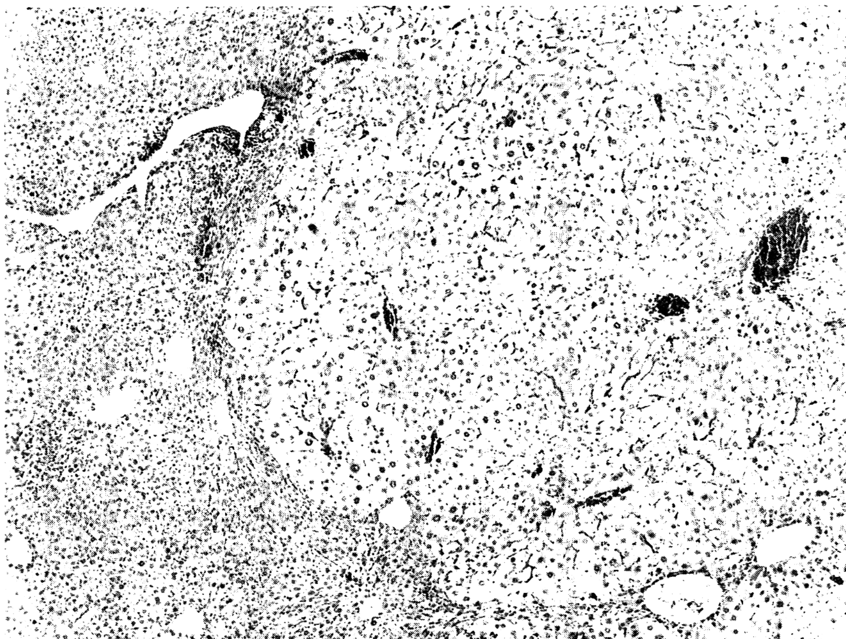

Fig. 4. Eosinophilic hepatocellular adenoma of a female mouse treated with 2200 ppm 2-nitro-*p*-phenylenediamine for 78 wk. The neoplasm has a more nodular appearance and shows compression of normal liver tissue. Haematoxylin and eosin ×54.

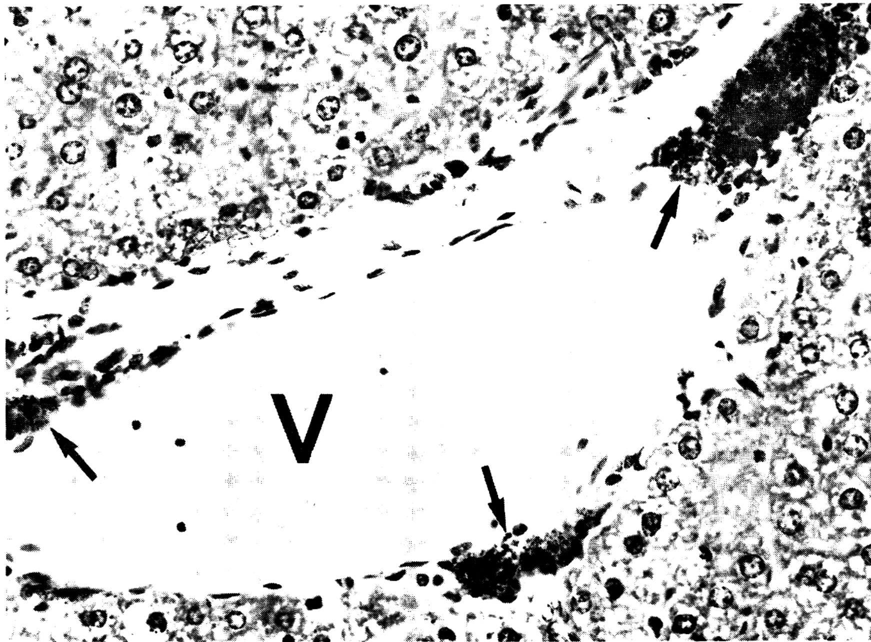

Fig. 5. Golden-brown pigment (arrowed) around a portal vein (V) × 330.

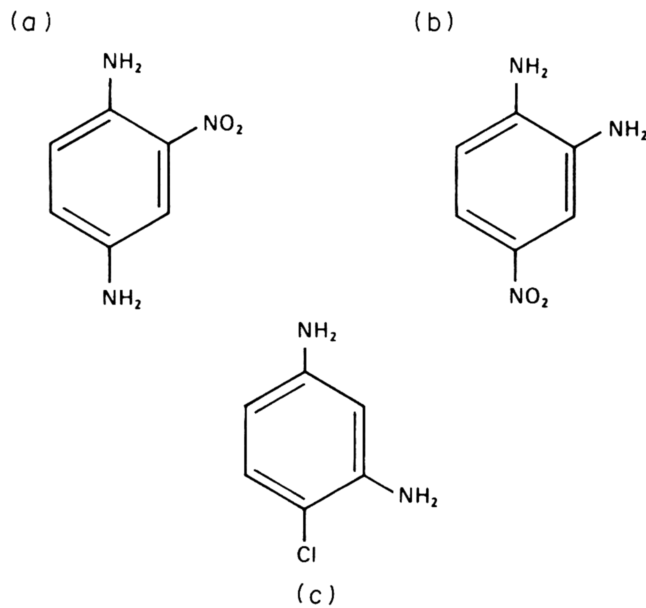

Fig. 6. Chemical structures of three aromatic amines used as dye components. (a) 2-nitro-*p*-phenylenediamine (b) 4-nitro-*o*-phenylenediamine and (c) 4-chloro-*m*-phenylenediamine.

no indication of a positive association between the administration of 4-NOPD and the increased tumour incidence. Treatment with 4-CMPD for 78 wk led to an increased incidence of hepatocellular neoplasms in female mice. Most of these tumours were small and they showed eosinophilic staining similar to that of the female mouse liver adenomas after 2-NPPD administration.

Hepatocellular adenomas induced by all three substances were morphologically similar. In female mice 2-NPPD and 4-CMPD were carcinogenic but 4-NOPD was not. A golden-brown pigment, which was not lipofuscin, was found in mice given 2-NPPD and 4-NOPD but not in those given 4-CMPD.

Hepatocytes with eosinophilic cytoplasm in induced hepatocellular adenomas have been reported by several authors (Butler & Hempsall, 1978; Frith & Dooley, 1976; Hoover, Ward & Stinson, 1979; Jones & Butler, 1975; Reuber, 1975; Rustia & Shubik, 1978). It has been demonstrated that large eosinophilic cells with abnormal nuclei and inclusions are characteristic of some compound-induced foci, hepatocellular adenomas and carcinomas (Jones & Butler, 1975) and that chemically-induced tumours may differ morphologically from those that arise spontaneously (Ward *et al.* 1979; Ward & Vlahakis, 1978).

In a 24-month study in which dogs were fed a composite material representative of a series of commercially available semi-permanent hair-colouring products. Wernick *et al.* (1975) found no gross or microscopic changes in various tissues including the liver in which neither were there any ultrastructural changes. But this study should be interpreted with caution because the number of test animals was low and only small amounts of the hair-dye mixture were used. Negative results were obtained in chronic studies of 2-NPPD and 4-NOPD with F344 rats, but 4-CMPD male rats showed an increased incidence of adrenal pheochromocytomas (National Cancer Institute, 1978; *ibid* 1979).

In these NCI studies it has been demonstrated that female mice fed the three structurally similar aromatic-amine dye components 2-NPPD, 4-NOPD and 4-CMPD develop liver tumours. These tumours are cytologically distinct from those which occur in untreated mice and treated males.

These findings indicate that mice are a valuable animal model in bioassay experiments despite their high incidence of spontaneous tumours. The incidence rate of such spontaneous neoplasms is well-known from previous research and so significantly increased incidences caused by carcinogen treatment can easily be detected by statistical methods. The extensive experience of pathologists with this species enhances the accuracy of morphological evaluation.

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SHORT PAPERS

DIHYDROAFLATOXIN INHIBITION OF ENERGY-LINKED REDUCTION OF ENDOGENOUS NICOTINAMIDE NUCLEOTIDES IN ISOLATED RAT-LIVER MITOCHONDRIA

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Abstract—Aflatoxins B_2 (AFB_2) and G_2 (AFG_2) have been evaluated for activity toward the energy-linked reduction of endogenous nicotinamide nucleotides by succinate and vitamin K_3 during anaerobic reversed electron transfer in isolated rat-liver mitochondria. These toxins induced a concentration-dependent biphasic inhibition involving three sites: succinate dehydrogenase, adenosine triphosphatase and respiratory complex I. While AFB_2 was more potent than AFG_2, it appeared that inhibition did not result from facilitation of proton ionophoresis.

Introduction

Aflatoxins B_2 (AFB_2) and G_2 (AFG_2) are bifuranocoumarin metabolites of the fungus *Aspergillus flavus* and are the corresponding dihydro derivatives of the more potent aflatoxins B_1 (AFB_1) and G_1 (AFG_1).

Results from previous studies have established that acutely toxic doses of AFB_1 and AFG_1 inhibit mitochondrial electron transport between cytochromes *b* and *c*, or c_1 (Doherty & Campbell, 1972 & 1973; Obidoa, 1975; Pai, Bai & Venkitasubramanian, 1975). AFB_2 and AFG_2 at sublethal doses appear to inhibit at the cytochrome-oxidase level (Obidoa, Bababunmi & Bassir, 1978). However, it has also been suggested that the degree and site(s) of inhibition by AFB_1 depend on the toxin concentration, the metabolic state of the mitochondria and the respiratory substrate used (Obidoa & Siddiqui, 1978).

It is relevant to mention here that the evidence from all but one available study seems to rule out any involvement of aflatoxin with electron- and energy-transfer processes in the region of the first phosphorylation coupling site. To clarify the situation further we have investigated the effects of both acutely toxic and sublethal (possibly carcinogenic) doses of AFB_2 and AFG_2 on the energy-dependent reduction of endogenous nicotinamide nucleotides by anaerobic reversed electron transfer across site 1 in isolated rat-liver mitochondria.

Experimental

Test solutions and reagents. AFB_2 and AFG_2 were purchased from Makor Chemical Products, Israel. They were dissolved in dimethylsulphoxide (DMSO) to produce a series of solutions of graded concentration. All common reagents were of the AnalaR grade. Vitamin K_3 was the product of F. Hoffman-La Roche and Co. Ltd., Basel, Switzerland.

Preparation of mitochondria. Mitochondria were isolated from the livers of male albino rats (body weight 250–300 g) by the method of Johnson & Lardy (1967). The mitochondrial pellet was washed twice with ice-cold 0·25 M-sucrose solution and was finally suspended in the same medium, to give a suspension containing about 10 mg mitochondrial protein/ml. All preparations were carried out at 0–4°C. All experiments with mitochondria were completed within a 10-hr period following the killing of the rats.

Determination of energy-linked nicotinamide nucleotide reduction by anaerobic reversed electron transfer

ATP-dependent reduction. Energy-linked reduction of endogenous nicotinamide nucleotides was determined by a modification of the spectrophotometric method of Sanadi (1963). The basal reaction medium (3 ml) containing about 4 mg mitochondrial protein was incubated for 2 min at 28 $\pm$ 2°C with 2 mM-potassium cyanide (to inhibit the activity of cytochrome oxidase), 30 mM-Tris buffer (pH 7·5), 1·3 mM-sodium succinate (pH 7·5) as an electron donor (or 1 μM-vitamin K_3 as 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester tetrasodium salt), and the appropriate AFB_2 or AFG_2 solution. An ATP solution (consisting of 2 mg bovine serum albumin, 2 mM-ATP and 3 mM-magnesium chloride) was then added to initiate the reaction. The reactants were mixed rapidly and placed in a spectrophotometer, and the absorbance changes at 340 nm were monitored for 10 min using an attached SP500 recorder.

Reduction dependent on high-energy intermediates (HEI). Systems of this second type involve, in principle, the oxidative generation of HEI (mitochondrial energized state) at one or more coupling sites and their utilization in reversing electron transport at another coupling site (Ernster, 1961). The method used was the same as that described for the ATP-dependent reduction except that the ATP solution

was omitted from the reaction system, and to energize the mitochondria, the suspension was aerated for about 15 min by means of an air pump. This method ensures that all the endogenous substrates are oxidized.

Controls and additional studies. Various control or reference reactions involving solutions containing 2,4-dinitrophenol (2,4-DNP; about 333 mM), DMSO or distilled water instead of aflatoxin solutions were also studied using both reduction systems. Additional experiments were carried out with mitochondria pre-incubated with each aflatoxin concentration for 10 min before the addition of other components of the reaction system.

Results

DMSO, the solvent for the aflatoxins, was used as the control in these experiments. However, in comparison with controls based on distilled water, DMSO caused some stimulation of both types of energy-linked reduction of nicotinamide nucleotides by either succinate or vitamin K_3, the stimulation being greater with the former substrate than with the latter. The inhibitory effects of the aflatoxin solutions and 2,4-DNP are therefore related to the raised values obtained with DMSO alone, the extent of this stimulation in each system being indicated by the apparent degree of inhibition by distilled water under comparable conditions (Table 1).

2,4-DNP, a classic respiratory uncoupler, caused severe (70–100%) inhibition of both types of reduction, irrespective of the substrate (Table 1). The degree of inhibition, however, was generally greater with succinate as the substrate, and probably indicated some inhibition (18%) of succinate-dehydrogenase activity. The dominant site of inhibition clearly occurred between complex I and the phosphorylation reaction sequence to 'X ~ I' (non-phosphorylated HEI; Table 1). This is consistent with the well-known effects of uncouplers on energy-dependent reversed electron transport.

At all concentrations tested, AFB_2 and AFG_2 markedly inhibited the anaerobic reduction of endogenous nicotinamide nucleotides by succinate irrespective of whether ATP or HEI was the energy source (Table 1). Inhibition was in all cases a direct function of aflatoxin concentration. Inhibition was generally more marked with the ATP- than the HEI-dependent reaction irrespective of the substrate (Table 1), possibly suggesting a slight inhibition between ATP and 'X ~ I' (oligomycin-sensitive site). Our data also suggest that a principal site of inhibition (15–60%) by these toxins at all concentrations (Table 2) was succinate dehydrogenase, the degree of inhibition being very slight when vitamin K_3 was the substrate. However, at the highest aflatoxin concentrations tested, a marked inhibition, comparable with that of succinate dehydrogenase, was observed between complex I and the phosphorylation reaction sequence to 'X ~ I'.

To establish whether AFB_2 and AFG_2 inhibited ATP translocation as atractylate does, mitochondria were pre-incubated with these toxins (1×10^{-7} M) prior to initiation of the reactions with either ATP or HEI. From the results presented in Table 3, it appears that these toxins had no effect on ATP translocation. Comparison of the data in Tables 1 and 3 suggests that the inhibitory effects of AFB_2 and AFG_2 may be partially (or totally) reversible with time. This possibility requires further experimental clarification however.

Discussion

These studies demonstrate that anaerobic energy-linked reduction of endogenous nicotinamide nucleotides involving the first phosphorylation coupling site was inhibited by both AFB_2 and AFG_2, irrespective of the source of electrons. The apparently dominant mechanisms involve a biphasic concentration-depend-

Table 1. *Effect of dihydroaflatoxins and 2,4-dinitrophenol on the inhibition of mitochondrial nicotinamide nucleotide reduction using succinate or vitamin K_3 as the substrate*

			Percentage inhibition*			
			Succinate substrate		Vitamin K_3 substrate	
Compound	Concn(M)	Reaction...	ATP-dependent	HEI-dependent	ATP-dependent	HEI-dependent
Water	—†		18 ± 2·8	31 ± 2·7	12·5 ± 1·6	10 ± 0·5
2,4-DNP	$0·83 \times 10^{-4}$		96 ± 5·6	90 ± 2·8	78 ± 3·5	74 ± 0·67
AFB_2	$1·05 \times 10^{-5}$		88 ± 2·9	73 ± 1·3	82 ± 0·76	60 ± 1·5
	$1·05 \times 10^{-6}$		80 ± 2·4	52 ± 3·3	19 ± 0·5	18 ± 0·23
	$1·05 \times 10^{-7}$		72 ± 3·5	45 ± 1·5	13 ± 1·1	14 ± 0·9
	$1·05 \times 10^{-8}$		63 ± 1·9	41 ± 2·8	10 ± 0·32	11 ± 1·1
	$1·05 \times 10^{-9}$		53 ± 2·7	31 ± 0·8	6 ± 0·15	9 ± 1·0
AFG_2	$1·00 \times 10^{-5}$		89 ± 2·1	73 ± 2·1	83 ± 0·8	79 ± 1·0
	$1·00 \times 10^{-6}$		66 ± 1·4	58 ± 3·4	52 ± 2·3	44 ± 2·8
	$1·00 \times 10^{-7}$		63 ± 1·0	40 ± 0·2	34 ± 0·3	19 ± 1·05
	$1·00 \times 10^{-8}$		58 ± 1·8	35 ± 3·4	12 ± 1·3	9 ± 0·6
	$1·00 \times 10^{-9}$		45 ± 3·1	27 ± 1·3	9 ± 0·5	8 ± 0·2

HEI = High energy intermediates 2,4-DNP = 2,4-Dinitrophenol AFB_2, AFG_2 = Aflatoxins B_2 and G_2
*Calculated on the degree of reduction effected in control incubations containing 25 μl DMSO/3 ml reaction medium.
†Incubations carried out in the presence of 25 μl distilled water instead of the equivalent volume of DMSO.
Values are means ± SEM of data derived from duplicate experiments carried out on three different mitochondrial preparations.

Table 2. *Statistical analysis of data given in Table 1*

Compound	Concn(M)	Changes in percentage inhibition			
		From ATP- to HEI-dependent RET		From succinate to vitamin K_3 substrate	
		With succinate	With vitamin K_3	With ATP	With HEI
2,4-DNP	0.83×10^{-4}	−6	−4	−18	−16
AFB_2	1.05×10^{-5}	−15	−22	−6	−13
	1.05×10^{-6}	−28	−1	−61	−34
	1.05×10^{-7}	−27	+1	−59	−31
	1.05×10^{-8}	−22	0	−53	−30
	1.05×10^{-9}	−22	−2	−40	−22
Mean*:	1.05×10^{-6} to 10^{-9}	-24.8 ± 3.2	-0.5 ± 0.8	-53.3 ± 9.6	-29.3 ± 5.1
AFG_2	1.00×10^{-5}	−16	−4	−6	+6
	1.00×10^{-6}	−8	−8	−14	−14
	1.00×10^{-7}	−23	−15	−19	−21
	1.00×10^{-8}	−23	−3	−46	−26
	1.00×10^{-9}	−18	−1	−36	−19
Mean*:	1.00×10^{-6} to 10^{-9}	-18 ± 7.0	-6.75 ± 6.2	-28.8 ± 13.5	-20 ± 4.9

HEI = High energy intermediates RET = Reversed electron transfer 2,4-DNP = 2,4-Dinitrophenol; AFB_2, AFG_2 = Aflatoxins B_2 and G_2

*Means are calculated on the four lower concentrations of each aflatoxin and are expressed as the mean ± SD.

Table 3. *Effect of mitochondrial pre-incubation with the test compound on the aflatoxin- or 2,4-DNP-induced inhibition of energy-linked reduction of mitochondrial nicotinamide nucleotide by succinate*

Compound	Concn(M)	Reaction...	Percentage inhibition*	
			ATP-dependent	HEI-dependent
2,4-DNP	0.83×10^{-4}		77 ± 0.08	64 ± 2.1
AFB_2	1.05×10^{-7}		11 ± 0.9	0
AFG_2	1.00×10^{-7}		31 ± 1.5	14 ± 0.3

HEI = High energy intermediates 2,4-DNP = 2,4-Dinitrophenol AFB_2, AFG_2 = Aflatoxins B_2 and G_2

*Calculated on the degree of reduction effected in control incubations containing 25 μl DMSO/3 ml reaction medium. Values are means ± SEM of data derived from duplicate experiments carried out on three different mitochondrial preparations pre-incubated in each case with 2,4-DNP or the aflatoxin for 10 min before addition of the other reactants, but otherwise comparable with the corresponding values in Table 1.

ent inhibition at three sites, namely succinate dehydrogenase, the step between respiratory complex I and the phosphorylation reaction sequence to 'X ~ I' and the reaction steps from ATP to 'X ~ I' (ATPase). At the highest test concentration, inhibition (60–90%) occurred at all three sites, although the involvement of the last-named site was only minor. Succinate dehydrogenase was the dominant site of inhibition (15–45%) at concentrations below 1×10^{-5} M. Generally AFB_2 was a more potent inhibitor than AFG_2.

In previous studies, redox measurements on respiratory complexes IV (cytochrome oxidase), V (glutamate–cytochrome *c* reductase) and VI (succinate–cytochrome *c* reductase) in the presence of AFB_2 (1.5×10^{-6} M) and AFG_2 (2×10^{-6} M), with and without pre-incubation, provided no evidence that these toxins could inhibit the activity of succinate dehydrogenase or the processes of electron- and energy-transfer reactions at site I (Obidoa *et al.* 1978). Rather, electron flow was inhibited terminally (cytochrome oxidase) and then only to a minimal extent. These discrepancies suggest that the underlying mechanisms operating in the conditions prevailing in these different studies were not directly related. The biochemical explanations for these different effects, therefore, may relate to the physicochemical characteristics (energetics and kinetics) of reversed electron transfer being favourable for the altered interactions between these toxins and the respiratory chain components. The inference appears consistent with contrasting effects of respiratory inhibitors like oligomycin and aurovertin on reversed electron transfer (Ernster & Lee, 1967).

Our results confirm that the well-known uncoupler, 2,4-DNP, is a potent inhibitor of energy-linked reversed electron transfer (Ernster, 1961). The major site of inhibition lies within complex I and the phosphorylation reaction sequence to 'X ~ I'. Since AFB_2 and AFG_2 exhibited a comparable effect only at concentrations of 1×10^{-5} M, it is logical to suggest that the dominant mechanism is different from that of DNP. It is probable that these toxins bind to one or more electron- or energy-transfer carriers rather than hydrolyse it (them) like DNP. In chemiosmotic terms they are probably not proton-ionophoretic. This conclusion supports our previous report that AFB_2 and AFG_2 do not stimulate ATPase activity or mitochondrial swelling and therefore do not uncouple oxidative phosphorylation (Obidoa *et al.* 1978).

Thus while the potency of AFB_2 is greater than that of AFG_2, the mechanisms of action of the two toxins are probably the same and a common structural feature may be responsible for their action. The evidence presented here strongly suggests that other aflatoxins (B_1, M_1 and G_1) may exhibit similar activity towards energy-linked reversed electron transfer between succinate and NAD^+, although this has not previously been demonstrated. Similarly it appears that whereas fungal coumarin-based metabolites probably do not interact with the energy conservation pathway (oxidative phosphorylation), they may do so when these reactions are reversed. We have recently concluded studies along these lines and the results will be presented elsewhere.

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EFFECT OF GRADED DOSES OF γ-IRRADIATION ON AFLATOXIN PRODUCTION BY *ASPERGILLUS PARASITICUS* IN WHEAT

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Abstract—The use of γ-irradiation can extend the storage life of certain foods, but following irradiation, some foods have been found, under laboratory conditions, to support a greater production of aflatoxin B_1. Wheat irradiated at different dose levels up to 250 krad showed a dose-dependent susceptibility to aflatoxin production, both toxin production and the free fatty acid levels of the wheat grains following an exponential pattern as the irradiation dosage increased from 50 to 250 krad. However, no correlation was observed between the degree of irradiation and the growth of the fungus.

Introduction

Aflatoxins are well-known toxic metabolites produced by some strains of Aspergillus, particularly *A. flavus* and *A. parasiticus*. The spores of these fungi are ubiquitously distributed and readily infest stored food-grains when a high humidity allied to a suitable temperature provide conditions conducive to their growth. The hepatotoxicity of aflatoxins in farm animals, poultry and primates, including man, is well documented (Butler, 1974; Newberne & Butler, 1969; Tulpule, Madhavan & Gopalan, 1964) and their carcinogenic potential has also been widely reported (Gopalan, Tulpule & Krishnamurthi, 1972; Swenson, Miller & Miller, 1974; Wogan, 1973; Wong & Hsieh, 1976).

The use of γ-irradiation has been considered an effective method of extending the storage life of certain foods by controlling microbial and parasitic activity (Bellamy, 1959; Hannesson, 1972). Although this technique has certain advantages, recent studies have suggested that freshly irradiated wheat may not be safe for human consumption and may need to be stored for at least 12 wk to render it wholesome and safe (Vijayalaxmi, 1975). Furthermore, an increase in the susceptibility of foods to fungal infestation after irradiation was suggested by Sommer & Fortlage (1966) and was attributed to a reduction in the physiological and biochemical resistance of the host tissue, leading to the rapid growth of some fungi if re-infection occurred as a result of unsatisfactory storage conditions. In this connection, the observation of Priyadarshini & Tulpule (1976) that aflatoxin production by *A. parasiticus* increased significantly in some agricultural commodities following γ-irradiation appears to be of practical importance. However, it was not clear from these studies whether the toxin production increased as a result of an increase in fungal growth or whether the increase was due to an increase in the production of toxin per unit growth of the fungus.

An investigation was undertaken, therefore, to study the effect of graded doses of γ-irradiation on aflatoxin B_1 production, free fatty acid levels and the growth of *A. parasiticus* in wheat, since it has been suggested that free fatty acid levels in foods change significantly after irradiation (Food Irradiation Information, 1975) and that fatty acids can support increased production of aflatoxin (Jemmali & Guilbot, 1974).

Experimental

A 4-day-old culture of *A. parasiticus* (NRRL 2999), maintained on potato-dextrose agar slants, was used as the inoculum (8×10^5 spores/ml). Normal, healthy wheat grains, in 50-g quantities, were irradiated at dose levels of 50, 75, 100, 200 and 250 krad using a ^{60}Co irradiation source. The dose delivered was checked by ferrous sulphate dosimetry. Duplicate 10-g samples of wheat irradiated at these five different levels and of non-irradiated wheat were sterilized by autoclaving at 15 psi for 15 min, inoculated with 1-ml aliquots of a uniform spore suspension of *A. parasiticus*, and incubated at 28°C for 7 days. After incubation, the material was sprayed with ethyl alcohol, dried at 70–80°C and powdered.

From each sample, a 100-mg aliquot was taken for the quantitative determination of fungal growth, glucosamine being estimated as a measure of chitin, by the procedure described by Priyadarshini & Tulpule (1978). The rest of the material was used for the extraction and estimation of aflatoxin (Stoloff, Nesheim, Yin, Rodricks, Stack & Campbell, 1971). The amount of aflatoxin B_1 was determined with a fluorodensitometer.

Free fatty acid levels were estimated, by the method of Lowry & Tinsley (1976), in each of the irradiated and non-irradiated batches of whole wheat.

Table 1. *Effect of graded doses of γ-irradiation on aflatoxin production, free fatty acid levels and the growth of* Aspergillus parasiticus *in wheat*

Irradiation dose (krad)	Aflatoxin B_1 (μg/g grain)	Free fatty acids (μg/g wheat flour)	Fungal weight (mg/g grain)
0	358 ± 30·7	354 ± 4·6	18·5 ± 1·40
50	512 ± 4·3	493 ± 0·5	20·2 ± 0·75
75	544 ± 6·2	532 ± 7·7	17·9 ± 0·94
100	556 ± 10·0	540 ± 0·7	18·6 ± 0·29
200	571 ± 9·1	570 ± 1·6	16·3 ± 2·09
250	633 ± 11·2	599 ± 2·2	16·3 ± 1·00

Values are means ± SEM ($n = 6$; samples from duplicate irradiations). There were no significant differences between duplicates and replicates for any parameter or between fungal growth determinations at different levels of irradiation. Both aflatoxin levels and the levels of free fatty acids differed significantly ($P < 0·05$, using an F ratio test of variance) at different levels of irradiation.

Results and Discussion

The results presented in Table 1 show that, compared with the non-irradiated grain, irradiated wheat supports the formation of significantly greater amounts of aflatoxin, under laboratory conditions. With increasing levels of irradiation from 50 to 250 krad, aflatoxin production was found to increase from 512 to 633 μg/g of grain. Non-irradiated wheat supported a mean aflatoxin production of 358 μg/g, and the minimum irradiation dose of 50 krad resulted in a 43% increase in aflatoxin production. As the irradiation dose increased, the aflatoxin production followed an exponential curve and consequently a logarithmic plot gave a linear relationship, with $r = 0·9891$.

Irradiation of wheat at all the levels studied caused an increase in the free fatty acid levels (Table 1) from 493 μg/g of grain with a dose of 50 krad to 599 μg/g with 250 krad. The lowest level of irradiation increased the free fatty acid level by about 40% over the non-irradiated control value of 354 μg/g.

As observed in the case of aflatoxin B_1 production, the free fatty acid levels followed an exponential curve as the irradiation dose increased from 50 to 250 krad, and the logarithmic plot gave a linear relationship with $r = 0·9794$, a very significant correlation.

Table 1 also indicates that irradiation of wheat at the dose levels studied did not alter the somatic growth of *A. parasiticus*. Fungal growth in terms of glucosamine varied within a narrow range of 16·3–20·2 mg/g of grain. No significant correlation was observed between the level of irradiation and the growth of *A. parasiticus* in wheat ($r = 0·4546$).

The results of this study confirmed earlier findings from this laboratory (Priyadarshini & Tulpule, 1976) that aflatoxin B_1 production is enhanced following exposure of wheat to γ-irradiation. Irradiation at dose levels of 50–250 krad increased both aflatoxin B_1 levels and free fatty acid levels in a similar and dose-dependent manner. However, no relationship was observed between levels of irradiation and the growth of the fungus, suggesting that the effect of irradiation on aflatoxin production in wheat is independent of the somatic growth of *A. parasiticus*. It is clear that irradiation of wheat does not alter the susceptibility of the grain to fungal infection but can bring about changes in the grain composition favouring an increased production of toxin.

It can be suggested, therefore, on the basis of these observations, that since aflatoxin production is accompanied by a concomitant increase in free fatty acid levels in the wheat, the levels of free fatty acids in a grain could be responsible to some extent for determining the amount of aflatoxin produced by the fungus. In fact, Jemmali & Guilbot (1974) have shown that lipids stimulate aflatoxin production. In other (unpublished) studies, we have demonstrated that saturated fatty acids (C 14:0, C 16:0 and C 18:0) stimulate aflatoxin synthesis in a synthetic medium, while unsaturated fatty acids tend to inhibit the toxin synthesis. In this context, the observation of Ba, Jemmali & Drapron (1977) that the toxigenic strains of *A. flavus* have a higher lipolytic activity than the non-toxigenic strains may be relevant. Investigations are in progress to study the role of different fatty acids on aflatoxin production, using natural substrates as well as synthetic media.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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ACETOIN

Synonyms: Acetylmethylcarbinol; 2-butanol-3-one; 3-hydroxy-2-butanone.
Structure: $CH_3 \cdot CH(OH) \cdot CO \cdot CH_3$.
Description and physical properties: *Merck Index* (1976).
Occurrence: Reported to be found in corn, wine, vinegar (from grape or other sources) honey, cocoa, butter and roasted coffee and in currant and strawberry aromas (*Fenaroli's Handbook of Flavor Ingredients*, 1975) and also in apple, banana, citrus fruits, pear, plum, raspberry, peas, tomato, mint, bread, cheese, milk, fish, chicken, meat, beer and whiskey (CIVO-TNO, 1977).
Preparation: From diacetyl by partial reduction, from butane-2,3-diol by oxidation with Sorbose bacterium or from sugar by bacterial fermentation; also produced by some fungi, notably Aspergillus and Penicillium strains (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

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

Analytical data: Gas chromatogram, RIFM no. 77–6; infra-red curve, RIFM no. 77–6.

Status

Acetoin was given GRAS status by FEMA (1965), is approved by the FDA for food use (GRAS) and was included by the Council of Europe (1974) in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on this compound. CAS Registry No. 513–86–0.

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977). A dose of approximately 14 g/kg given sc was lethal to rats, causing paralysis and convulsions (Gaunt, Brantom, Kiss, Grasso & Gangolli, 1972).

Short-term toxicity. When acetoin was administered to rats in the drinking-water at levels of 750, 3000 and 12,000 ppm for 13 wk (Gaunt *et al.* 1972), no effects were seen at the 750 or 3000-ppm levels. At 12,000 ppm the rate of body-weight gain was reduced in association with a reduction in food and water consumption, the relative liver weight was increased without histological change, and there was a slight anaemia. The no-untoward-effect level of 3000 ppm was equivalent to an intake of approximately 330 mg/kg/day or almost 700 times the calculated maximum daily intake in man. The maximum tolerated dose (MTD), defined as the maximum single dose tolerated by all five mice given six ip injections over a 2-wk period, was found to be 3·00 g/kg (Stoner, Shimkin, Kniazeff, Weisburger, Weisburger & Gori, 1973).

Irritation. Acetoin applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1977). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 31 volunteers. The material (RIFM no. 77–6) was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Epstein, 1977).

Metabolism. When acetoin was fed to dogs, some 5–25% of the dose was excreted as butane-2,3-diol, together with small amounts of the unchanged compound (Williams, 1959). Injection into decere-

*The most recent of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology*, 1979, **17**, no. 4 (pp. 357–390).

brate cats was followed by considerable production of butane-2,3-diol, but injection of this glycol did not give rise to appreciable amounts of acetoin (Dawson & Hullin, 1954). In reviews of the metabolism of acetoin, Gabriel, Ilbawi & Al-Khalidi (1972) and Gaunt *et al.* (1972) referred to the urinary excretion of small amounts of acetoin and elevated levels of butane-2,3-diol in acetoin-fed dogs, increased acetylation in acetoin-fed rabbits and in rabbit-liver homogenate incubated with acetoin and some incorporation into sterols in rat-liver slices incubated with acetoin.

Acetoin was detected as an intermediary metabolite in the reduction of methyl ethyl ketone to butane-2,3-diol in male guinea-pigs and was cleared in 16 hr (DiVincenzo, Kaplan & Dedinas, 1976). In albino rats injected with near-physiological blood levels of acetoin, a major fraction was oxidized to CO_2 and relatively small amounts were reduced to butane-2,3-diol (Gabriel *et al.* 1972). Oxidation probably occurred through formation of acetate units. In these rats, [2,3-^{14}C]acetoin conversion to $^{14}CO_2$ in the expired air was 47.7% after 20 hr following intracardial injection and 15% after 12 hr following ip injection. Up to 22% of the radioactivity was excreted in the urine within 18 hr as unidentified compounds. The absence of glycogen formation was demonstrated in albino mice injected ip with 200 mg acetoin in two doses (Gabriel *et al.* 1972).

Acetoin is sometimes formed together with butane-2,3-diol during the fermentation of sugars by *Escherichia coli* and other members of the Bacteriaceae (Williams, 1959).

Carcinogenicity. In an assay for carcinogenicity, A/He strain mice received 20 ip injections of acetoin three times per week in total doses of 60·0 g/kg (MTD) and 12·0 g/kg ($\frac{1}{5}$ MTD) (Stoner *et al.* 1973). After 22 wk, Pseudomonas infection was present but there was no increased incidence of primary lung tumours or abnormality of other organs and the compound was not considered carcinogenic.

Pharmacology. When acetoin was administered iv to cats, EEG changes in various regions of the brain were noted (Reith, Holm, Staiger, Urbanek & Lucman, 1975). When acetoin was administered ip to normal, partially hepatectomized or hepatically damaged rats, the major neurological effect was a disturbance of consciousness ranging from apathy to somnolence (Nishikava, 1970). Male albino rats receiving $5{\cdot}5 \times 10^{-3}$ mol acetoin/kg ip showed no behavioural changes, no anaesthetic response and no deviation from normal in EEG tracings (Sprince, Josephs & Wilpizeski, 1966).

Enzymes. Cell-free extracts of rat liver oxidized acetoin to CO_2 in the presence of a mitochondrial fraction, ATP and NAD (Ibrahimi, Gabriel & Al-Khalidi, 1972).

Isolated tissues. Acetoin in concentrations $\leq$ 5 mM did not affect protein synthesis in rat-liver slices (Perin, Scalabrino, Sessa & Arnaboldi, 1974). Bloom, Fuller, Westerfeld & Westerfeld (1966) reported that acetoin was not catabolized by various types of rat-tissue homogenates, but Gabriel, Jabara & Al-Khalidi (1971) found that with high concentrations of acetoin (4·5 mM), up to 70% was reduced to butane-2,3-diol in albino rat-liver mince, and Gabriel *et al.* (1972) demonstrated the production of $^{14}CO_2$ from [2,3-^{14}C]acetoin in rat-liver mince, the rate of this $^{14}CO_2$ production being about five times slower than from [1,4-^{14}C]acetoin. Production of $^{14}CO_2$ from labelled acetoin was depressed by certain inhibitors of the tricarboxylic acid cycle and by acetate (Gabriel *et al.* 1972).

Isolated cat brains exhibited alterations in EEG tracings when treated with acetoin (Reith *et al.* 1975).

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AMYL ISOEUGENOL

Synonyms: Isoeugenol amyl ether; 1-pentoxy-2-methoxy-4-propenylbenzene.
Structure: $CH_3 \cdot CH{:}CH \cdot C_6H_3(OCH_3) \cdot O \cdot [CH_2]_4 \cdot CH_3$.
Description and physical properties: A pale-yellowish viscous liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From isoeugenol in alcoholic potassium hydroxide solution with amyl bromide, or by isomerization of amyl eugenol (Arctander, 1969).
Uses: Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·3
Maximum	0·2	0·02	0·05	0·8

Analytical data: Gas chromatogram, RIFM no. 75–168.

Status

Amyl isoeugenol is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974) or in the *Food Chemicals Codex* (1972). CAS Registry No. 10484–36–3.

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1976).

Irritation. Amyl isoeugenol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1976). Tested at 8% in petrolatum it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 75–168) was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Epstein, 1976).

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Moreno, O. M. (1976). Report to RIFM, 7 October.

BUTYL ACETATE

Synonym: *n*-Butyl acetate.
Structure: $CH_3 \cdot OCO \cdot [CH_2]_3 \cdot CH_3$.
Description and physical properties: *Merck Index* (1976).
Occurrence: Reported to have been found in rum ether (*Fenaroli's Handbook of Flavor Ingredients*, 1975) and in apple, banana, cherry, citrus fruits, currants, grape, melon, papaya, peach, pear, plum, raspberry, strawberry, tomato, vinegar, cheese, beer, brandy, wine, cocoa, coffee, roasted nuts and honey (CIVO-TNO, 1977).
Preparation: By direct esterification of *n*-butyl alcohol with acetic acid or acetic anhydride (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 8000 lb/yr.

Concentration in final product (%):

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

Analytical data: Gas chromatogram, RIFM no. 76–26; infra-red curve, RIFM no. 76–26.

Status

Butyl acetate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515), and was listed by the Council of Europe (1974) with an ADI of 1 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on butyl acetate. CAS Registry No. 123–86–4.

Biological data

Acute toxicity. The oral LD_{50} in rabbits was reported as 7·4 g (64 mmol)/kg (Munch, 1972) and the oral low lethal dose in rabbits and guinea-pigs as 3·2 and 4·7 g/kg, respectively (National Institute for Occupational Safety and Health, 1976). In rats, the oral LD_{50} was found to be 14 g/kg (National Institute for Occupational Safety and Health, 1976), and in mice 7·1 g/kg (Browning, 1965).

A dose of 750 mg butyl acetate/kg injected ip into mature male guinea-pigs was accompanied by a slight increase in serum ornithine carbamyl transferase activity; when the dose was increased to 1500 mg/kg, two of the four animals tested died and the mean serum ornithine-carbamoyltransferase activity of the surviving guinea-pigs was elevated (DiVincenzo & Krasavage, 1974). Histological examinations suggested a slight accumulation of lipid in the liver cells. The ip LD_{50} for butyl acetate in mice was 1·23 g/kg (National Institute for Occupational Safety and Health, 1976). The ip toxicity in rats has been related to hydrolysis of the ester (Selisko, Ackermann & Kupke, 1962). The acute dermal LD_{50} in rabbits was reported as > 5 g/kg (Moreno, 1976).

Inhalation. A review of the physiological effects of butyl acetate exposure in man indicates anaesthetic action at high concentrations and slight eye irritation at lower concentrations (Fassett, 1963). Brief exposure to 14,000, 7000 or 3300 ppm butyl acetate was found to be disagreeable to human subjects because of a strong odour and irritant effects on the eyes and nose (Browning, 1965). Lower concentrations, between 200 and 300 ppm, caused slight eye irritation but no corneal oedema (Browning, 1965; Fassett, 1963). Throat irritation has been reported upon exposure to 200 ppm butyl acetate in an occupational setting; the irritation became quite severe at 300 ppm, and a threshold limit value of 150 ppm has been recommended (American Conference of Governmental Industrial Hygienists, 1971; National Institute for Occupational Safety and Health, 1976). This value was determined on an 8 hr/day, 40 hr/wk basis over a normal working lifetime (Rosensteel, 1974; Ruhe, 1974).

Cats exposed to 83 mg butyl acetate/litre for 30 min experienced narcosis and death, while 55 mg/litre for 30 min caused only narcosis; cats inhaling 20 mg/litre for 6 hr on six consecutive days experienced weakness, weight loss and minor blood changes (Fassett, 1963).

In guinea-pigs, a concentration of 14,000 ppm butyl acetate was dangerous to life and the maximum concentration that could be sustained for 1 hr without serious disturbance was 7000 ppm (Browning, 1965). Inhalation of 67 mg/litre of air for 4 hr caused eye irritation, narcosis and death in guinea-pigs, and inhalation of 33 mg/litre for 13 hr had similar effects but the animals recovered (Fassett, 1963). At 16 mg/litre over 13 hr, eye irritation was the only effect observed. Inhalation of 35 mg butyl acetate/litre of air for 3 hr caused narcosis in mice (Fassett, 1963). Upon repeated inhalation of 3100–4200 ppm for 6 hr/day for 6 days, mice became habituated to the irritation but showed some fatigue and weight loss; blood changes included an increase in the formed elements and in haemoglobin concentration (Browning, 1965).

Congestion of lungs, brain, liver, and renal tubules, as well as slight pulmonary emphysema, was observed in animals exposed to butyl acetate (Browning, 1965).

Irritation. *n*-Butyl acetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1976). Butyl acetate may cause conjunctivitis in man

(*Merck Index*, 1976). An eye splash of butyl acetate was healed in 48 hr (Browning, 1965). Tested at 4% in petrolatum, butyl acetate produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 26 volunteers. The material (RIFM no. 76-26) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1976).

Teratogenesis. Chicken eggs injected with a dose of 45 mg butyl acetate/egg did not hatch; when 27 mg/egg was injected, 45% of the eggs hatched and when 9 mg/egg was injected 60% of the eggs hatched (McLaughlin, Marliac, Verrett, Mutchler & Fitzhugh, 1964). Kidney damage and corneal lesions were observed in the chick embryos.

Pharmacology. The narcotic dose (ND) for guinea-pigs was 10,000-14,000 ppm inhaled for 15-30 min (Browning, 1965). The ND_{50} after oral administration to rabbits was 2·2 g (19 mmol)/kg and the threshold narcotic concentration to tadpoles was 0·7 g (6 mmol)/litre water (Munch, 1972). Anaesthetic effects were not observed in humans exposed to butyl acetate for 2 or 3 hr at levels of 400-600 ppm (Fassett, 1963).

Cells. *In vitro* exposure of Ehrlich-Landschütz diploid ascites tumour cells to 50 or 100 ppm butyl acetate for 1-5 hr produced only slight cytotoxicity (Holmberg & Malmfors, 1974). Incubation of rabbit polymorphonuclear leucocytes with certain phosphonate esters inhibited cell response to the chemotactic factor; this inhibition could be prevented by addition of butyl acetate to the incubation medium (Becker & Ward, 1967 & 1968; Ward & Becker, 1968). Butyl acetate exhibited an antihaemolytic effect on erythrocytes undergoing hypotonic haemolysis (Holmberg, Jakobson & Malmfors, 1974).

Isolated tissue. Butyl acetate, combined with choline, exhibited a contractile effect on isolated guinea-pig ileum (Ishida, Moritoki & Akira, 1969). In this tissue, butyl acetate elicited contractions that could be inhibited by cooling, procaine hydrochloride or atropine sulphate; release of acetylcholine by butyl acetate was indicated (Takagi & Takayanagi, 1966), and when butyl acetate combined with muscle acetylcholine receptor, the response to acetylcholine was inhibited. Butyl acetate was hydrolysed by rat liver and intestine as well as by artificial gastric and pancreatic juices (Longland, Shilling & Gangolli, 1977). The stimulation of frog olfactory epithelium by butyl acetate has been studied (Minor & Sakina, 1973; Mozell, 1969).

Enzymes. Butyl acetate did not affect the activity of rat mast-cell protease in the presence of di-isopropyl fluorophosphate (Darzynkiewicz & Barnard, 1967), but inhibited rat-liver monoamine oxidase (Gomi, 1966) and was a weak inhibitor of bovine carbonic-anhydrase activity (Pocker & Stone, 1968). Moderate increases in leucocyte peroxidase and cytochrome-oxidase activities, as well as a marked increase in the activity of alkaline phosphatase, were observed in industrial workers who were exposed to butyl acetate among other solvents (Dinca, Pod, Galetariu & Budisca, 1969).

Effects of mixtures containing butyl acetate. Inhalation of a paint thinner containing acetate esters, toluene and xylene caused drunkenness and hallucination in human subjects (Tambo, 1973). In an attempt to characterize the toxic effects of this thinner, a mixture of the five acetates, including butyl acetates, found in it was administered orally to rabbits. Administration was accompanied by a decrease in P_{CO_2} and P_{O_2} in the blood. Gas chromatography revealed that the esters were hydrolysed to acetic acid and the corresponding alcohol. The alcohols caused drunkenness and the acetic acid caused acidosis in the blood (Tambo, 1973).

Blood chemistry was performed on workers who were exposed to a variety of fat solvents. Normocytic normochromic anaemia occurred and was attributed to exposure to butyl acetate and butyl alcohol; plasma bicarbonate was also lowered because of liberation of acetic acid (Shalaby, El-Danasory, Massoud & Hathout, 1973). Nail perspiration was strongly decreased when a cosmetic varnish containing butyl acetate was applied to the nails (Jacobi, 1968). The acute oral LD_{50} values for several varnish thinners containing butyl acetate ranged from 7·6 to 8·6 mg/kg in mice (Senczuk, Orlowski & Jodynis, 1977).

In other studies of groups of workers, mixtures containing butyl acetate have been shown to affect behaviour on the one hand (Binaschi, Gazzaniga & Crovato, 1976) and to be nontoxic on the other (Rosensteel, 1974; Ruhe, 1974).

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BUTYL *n*-BUTYRATE

Synonym: *n*-Butyl *n*-butanoate.
Structure: $CH_3 \cdot [CH_2]_3 \cdot OCO \cdot [CH_2]_2 \cdot CH_3$.
Description and physical properties: *Merck Index* (1976).
Occurrence: Reported to be found in apple, banana, grape, pear, strawberry, plum and passion fruit (CIVO-TNO, 1977).
Preparation: By direct esterification of *n*-butyl alcohol with *n*-butyric acid under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 2000 lb/yr.

Concentration in final product (%):

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

Analytical data: Gas chromatogram, RIFM no. 77–49; infra-red curve, RIFM no. 77–49.

Status

Butyl butyrate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was listed by the Council of Europe (1974) with an ADI of 1 mg/kg. Monographs on butyl butyrate have been prepared by Browning (1965) and for the *Food Chemicals Codex* (1972). CAS Registry No. 109–21–7.

Biological data

Acute toxicity. The LD_{50} of butyl butyrate administered to rabbits by stomach tube was 9·5 g/kg (66 mmol/kg) (Munch, 1972). The ip LD_{50} was approximately 2·3 g/kg for male rats (Selisko, Ackermann & Kupke, 1962) and 8·9 g/kg for mice (Sporn & Dinu, 1965). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1977).

Subacute toxicity. When rats were treated orally with butyl butyrate for several weeks, the activities of succinic dehydrogenase and glutamic–aspartic transaminase decreased, while the activity of aldolase increased in the liver (Sporn & Dinu, 1965).

Irritation. Butyl *n*-butyrate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1977). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–49) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1977).

Pharmacology. The ND_{50} (narcotic dose) for butyl butyrate administered to rabbits by stomach tube was 5·2 g/kg (36 mmol/kg), the higher doses being associated with loss of corneal reflex, nystagmus, dyspnoea and bradycardia (Munch, 1972). When the anti-inflammatory action of butyl butyrate was investigated during ip administration of 50 mg/kg to rats, the ester was found to be less effective than butyric acid in inhibiting the development of dextran-induced oedema (Ladinskaya, 1968).

Nutrition. When *n*-butyl *n*-butyrate was fed to chicks at 5% in the diet, it was palatable but only 44% of its energy was available (Yoshida, Morimoto & Oda, 1970).

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CINNAMYL ISOBUTYRATE

Structure: $C_6H_5 \cdot CH{:}CH \cdot CH_2 \cdot OCO \cdot CH(CH_3) \cdot CH_3$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of cinnamic alcohol with isobutyric acid under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.

Concentration in final product (%):

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

Analytical data: Gas chromatogram, RIFM no. 77–76; infra-red curve, RIFM no. 77–76.

Status

Cinnamyl isobutyrate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) at a level of 20 ppm (except for chewing gum) in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. CAS Registry No. 103–59–3.

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977).

Irritation. Cinnamyl isobutyrate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Moreno, 1977). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 31 volunteers. The material (RIFM no. 77–76) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1977).

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CITRONELLYL NITRILE

Structure: $CH_3 \cdot (CH_3)C{:}CH \cdot [CH_2]_2 \cdot CH(CH_3) \cdot CH_2 \cdot CN$.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By treatment of citronellyl halide with potassium cyanide or by reacting citronellyl oxime with acetic anhydride (P.Z. Bedoukian, personal communication to RIFM, 1977).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·003	0·0025	0·08
Maximum	0·06	0·03	0·02	0·6

Status

Citronellyl nitrile is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974) or in the *Food Chemicals Codex* (1972). CAS Registry No. 52671-32-6.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 5·3 g/kg (4·2–6·7 g/kg) and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977).

Irritation. Citronellyl nitrile applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Moreno, 1977). Tested at 6% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77-87) was tested at a concentration of 6% in petrolatum and produced no sensitization reactions (Kligman, 1977).

References

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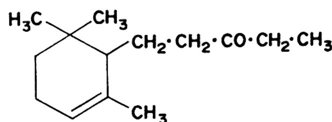
DIHYDROMETHYL-α-IONONE

Synonym: 5-(2,6,6-Trimethyl-2-cyclohexenyl)-pentan-3-one.
Structure:



Description and physical properties: A colourless oily liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By controlled catalytic hydrogenation of α-methylionone (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·2
Maximum	0·1	0·01	0·03	0·8

Analytical data: Gas chromatogram, RIFM no. 76–79; infra-red curve, RIFM no. 76–79.

Status

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

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Bailey, 1976).

Irritation. Dihydromethyl-α-ionone tested at 4% in petrolatum produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 76–79) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1976).

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FENNEL OIL, BITTER*

Since publication of an earlier monograph* on fennel oil, bitter, additional work with different samples has demonstrated that the original sample had deteriorated because of atmospheric oxidation. Signs of excessive deterioration included the presence of anisic aldehyde at levels above 0·3% and of *p*-cymene at levels above 2%. Acceptable samples of fennel oil, bitter should not contain more than 0·3% anisic aldehyde or 2% *p*-cymene as determined by GLC.

Occurrence: Found in the seeds of *Foeniculum vulgare* Miller subsp. *capillaceum* (Gilib.) Holmboe var. *vulgare* (Miller) Thellung, a wildgrowing type, sometimes known as bitter fennel (Gildemeister & Hoffman, 1961).

Preparation: By steam distillation of the crushed seeds.

Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%).

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

Analytical data: Gas chromatogram, RIFM no. 77–DBC; infra-red curve, RIFM no. 77–DBC.

Status

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

Biological data

Irritation. Tested at 4% in petrolatum, fennel oil, bitter produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1978).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 29 volunteers. The material (RIFM no. 77–DBC) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1978).

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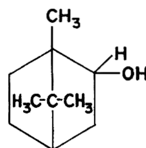
*Opdyke, *Fd Cosmet. Toxicol.* 1976, **14**, 309.

ISOBORNEOL

Synonyms: Isobornyl alcohol; isocamphol.
Structure:



Description and physical properties. EOA Spec. no. 250.
Occurrence: Isoborneol rarely occurs in nature but it is found in the oil of *Abies sibirica* and a few other essences and is probably present in the essential oil from the roots of *Chamaecyparis formosensis* (*Fenaroli's Handbook of Flavor Ingredients*, 1975).
Preparation: By the hydrolysis of isobornyl acetate.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·005	0·002	0·08
Maximum	0·15	0·015	0·05	0·3

Analytical data: Gas chromatogram, RIFM no. 77–244; infra-red curve, RIFM no. 77–244.

Status

Isoborneol was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515), and was included by the Council of Europe (1974) in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. CAS Registry No. 124–76–5.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 5·2 g/kg (4·3–6·2 g/kg) and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977).

Irritation. Isoborneol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1977). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 26 volunteers. The material (RIFM no. 77–244) was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Epstein, 1977).

Isolated tissue. Isoborneol was formed in very small amounts when *d*-camphor was reduced in rabbit-liver cytosol in an NADPH-dependent reaction (Leibman & Ortiz, 1973).

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p-ISOPROPYL PHENYLACETALDEHYDE

Synonyms: Cuminic acetaldehyde; 4-isopropyl phenylacetaldehyde; cumyl acetaldehyde; *p*-cymene-7-carboxaldehyde.
Structure: $CH_3 \cdot (CH_3)CH \cdot C_6H_4 \cdot CH_2 \cdot CHO$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From cumyl magnesium chloride plus ethyl formate or triethyl orthoformate, followed by acid hydrolysis of the acetal (Arctander, 1969).
Uses: In public use since the 1960s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.

Concentration in final product (%):

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

Status

p-Isopropyl phenylacetaldehyde was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) at a level of 0·5 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. CAS Registry No. 4395-92-0.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 4·1 g/kg (3·4–5·0 g/kg) and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977).

Irritation. *p*-Isopropyl phenylacetaldehyde applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was severely irritating (Moreno, 1977). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 76–344) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1977).

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Environmental Carcinogens—Selected Methods of Analysis. Vol. 1—Analysis of Volatile Nitrosamines in Food. Edited by R. Preussmann, M. Castegnaro, E. A. Walker and A. E. Wassermann. IARC Scientific Publications no. 18. International Agency for Research on Cancer, Lyon, 1978. pp. xiii + 212. Sw.fr. 90.00.

This publication marks the start of a series intended to provide a manual of selected methods for the analysis of environmental carcinogens. Volumes on vinyl chloride and polycyclic hydrocarbons are in preparation and this first volume is concerned with the analysis of volatile nitrosamines in food.

The volume is divided into three sections comprising a number of chapters contributed by individuals or groups of experts in this field. The first section contains a brief review of the carcinogenicity of *N*-nitroso compounds in experimental animals and its relevance to man. The exposure of the general population to *N*-nitroso compounds present in the environment and to these compounds when formed from precursors *in vivo* is also outlined.

The second section is a review of approaches to the analysis of both nitrosamines and their precursors and contains numerous references. Different sampling, extraction and clean-up techniques are described and their relative merits are discussed. Determination by polarography, spectrophotometry and various chromatographic techniques is dealt with similarly. Combined gas chromatography and mass spectrometry (GC–MS) is considered in some detail and it is concluded that the most reliable procedure for quantitative detection of nitrosamines is GC separation followed by MS using parent ion monitoring and peak matching at high resolution. Determinations by derivatization and the use of chemiluminescence-based detectors are also considered and analytical methods for the determination of precursor amines and of nitrate and nitrite in foodstuffs are reviewed.

The final section deals in great detail with eleven selected methods for analysing volatile nitrosamines in food. The methods are presented in ISO format, with the procedures written in discrete steps and with diagrams of apparatus where appropriate. The nitrosamines are generally isolated by distillation and extracted with dichloromethane. In the majority of methods free nitrosamines are determined, but two methods analyse for nitramines, and in another heptafluorobutyramide derivatives of nitrosamines are determined. In most methods determination is by GC followed by confirmation by GC–MS. This section is very useful.

Environmental Aspects of N-Nitroso Compounds. Proceedings of a Working Conference held at the New England Center for Continuing Education, University of New Hampshire, Durham, New Hampshire, USA. 22–24 August 1977. Edited by E. A. Walker, M. Castegnaro, L. Griciute and R. E. Lyle. IARC Scientific Publications no. 19. International Agency for Research on Cancer, Lyon, 1978. pp. xxiv + 566. Sw.fr. 100.00.

This may be regarded as a companion book to the IARC publication on methods of analysis for volatile nitrosamines in food (IARC Scientific Publications no. 18; see above) and its presentation is equally clear. It is divided into six sections covering the analysis, chemistry and formation, occurrence and precursors, biology and safety of nitrosamines, and the recommendations of the subcommittees that discussed the papers presented at the conference.

In the section on analysis, some papers describe generally applicable procedures whilst others are more specific; for instance, one compares two methods of determining volatile *N*-nitrosamines in cured meat products. The use of the thermal energy analyser (TEA) in conjunction with gas–liquid chromatography (GLC) and high-pressure liquid chromatography (HPLC) receives detailed attention. Amongst other techniques described are thin-layer chromatography (TLC), using fluorescamine, and nuclear magnetic resonance (NMR) spectrometry.

The topics dealt with in the section on chemistry and formation include the electronic properties of nitrosamines, fragmentation reactions of β-hydroxynitrosamines and transnitrosation. Studies *in vitro* on mechanisms of formation of nitrosamines are described and their relevance to possible mechanisms of formation in environmental media or *in vivo* is discussed. The effects of antibiotics, antioxidants and micro-organisms on nitrosamine formation are considered. One paper draws attention to the inadvertent formation of nitrosamines during analytical procedures.

Human exposure to environmental nitrosamines is considered in the section on occurrence and precursors, and papers concerning the presence of nitrosamines in food, tobacco smoke and pesticides are included. Trends in the levels of *N*-nitrosopyrrolidine in bacon from 1971 to 1977, and determinations of the emission of nitrosamines into the environment from factories producing or using amines are described. Included in the biology section are reports of studies on the relationship between the intake of dietary nitrate and nitrite, and levels of salivary nitrite and urinary nitrosamines, and between urinary infection and the production of nitrosamines in the bladder.

There is a short section on safety aspects of nitrosamine experimentation which warns that the protective effect of laboratory gloves is limited and advises that gloves should be used only once and discarded immediately if they become contaminated. Finally, in

their reports, the subcommittees that discussed the papers presented at the conference recommend guidelines for future research.

Like earlier IARC publications on *N*-nitroso compounds (*Cited in F.C.T.* 1978, **16**, 65), this is an excellent book and further volumes are awaited with interest.

Micro-organisms in Foods 1: Their Significance and Methods of Enumeration. 2nd Ed. International Commission of Microbiological Specifications for Foods. University of Toronto Press, Toronto, 1978. pp. xvii + 434. $30.00.

The assessment of the microbiological safety of foods has become increasingly important in recent years with the development of new techniques for the large-scale production, processing, preservation and storage of foods. Furthermore, the expansion of the international distribution of foods, especially from areas where enteric diseases are endemic, has increased the need for stringent microbiological controls.

The ICMSF was set up in 1962 to provide authoritative guidance on the microbiological testing of foods, in particular on the significance of the presence of particular species or groups of micro-organisms, and to try to establish internationally acceptable microbiological criteria. One of the first steps in that direction was the publication of the first edition of this book. The rapid progress in food microbiology, however, has made the revision of that edition essential, and it is a reflection of the extent of that progress that, in the process, the book has doubled in size.

The second edition encompasses the new developments in methodology for detecting and evaluating the presence of pathogens in food. Particular attention is paid to Salmonella and also to the results of recent work on the mode of action of *Bacillus cereus*, *Vibrio parahaemolyticus*, *Clostridium perfringens* and *Vibrio cholerae*. Also included in the new edition are sections on parasitic protozoa, helminths, haemolytic streptococci, food-spoilage organisms and food-borne viruses.

The book is divided into three parts. In part 1, the significance of the presence in food of various organisms and microbial toxins is discussed. Part 2 deals with methods recommended for the examination of micro-organisms associated with food-borne diseases and part 3 gives detailed information on the media and reagents used in the analyses.

This book can be highly recommended as a work of reference and a laboratory manual for people working in any aspect of food microbiology. The information is very lucidly and logically presented and there is an extensive bibliography.

Occupational Cancer—Prevention and Control. I.L.O. Occupational Safety and Health Series no. 39. International Labour Office, Geneva, 1978. pp. 36. Sw.fr. 10.00.

Following meetings in 1973 and 1974, the International Labour Conference adopted a Convention and a Recommendation concerning the prevention and control of occupational hazards caused by carcinogens. This booklet is intended to provide guidance on the implementation of principles set forth in these two documents, which are included as appendices. Topics discussed are problems relating to the establishment of occupational standards for carcinogens, classification of carcinogens for the purpose of legislation, preventive measures, exposure monitoring, personnel administration, medical supervision, and registers and recordings.

One conclusion reached is that it is extremely difficult to establish "safe" exposure levels for carcinogens by extrapolation from animal or human data, and that exposure should always be kept as close to zero as possible. Contact with 2-naphthylamine, dialkylnitrosamines, benzidine, 4-aminodiphenyl, 2-acetylaminofluorene, 2-nitronaphthylamine, 4-dimethylaminoazobenzene, 4-nitrodiphenyl, methylnitrosourea and bis(chloromethyl) ether should be avoided altogether. Stringent protective measures should be applied to limit exposure to 1-naphthylamine, propane sultone, asbestos, vinyl chloride, ionizing radiation and radioactive substances, methyl chloromethyl ether, diazomethane, 1,1-dimethylhydrazine, benzene and β-propiolactone. For a third group of substances (inorganic arsenic, nickel carbonyl, 4,4'-methylene-bis-*o*-chloroaniline, dimethyl sulphate, 3,3'-dichlorobenzidine, *o*-tolidine, dianisidine, ethyleneimine and ethylene thiourea) exposure should be kept to a minimum through the use of the most feasible and applicable controls. Similar controls are recommended for minimizing exposure to nine specified industrial processes, which are known to involve a significant carcinogenic risk, and technical and personal protective measures should be used in the case of several materials of complex composition (all containing polycyclic aromatic hydrocarbons) which have caused cancer in man.

Safety in Working with Chemicals. By M. E. Green and A. Turk. Collier Macmillan Publishers, London, 1978. pp. ix + 166. £2.75.

In view of the present concern over health and safety at work, the above monograph is a timely publication. It is concerned with the use of chemicals in all laboratories and aims to provide an understanding of safe practice and so reduce the incidence of laboratory accidents.

The authors feel that a correct attitude towards the working environment is the only way that accidents can be cut to a minimum. The use of protective clothing and eye shields and the maintenance of high standards of hygiene are among the obvious precautions long accepted as desirable, but dangerous short cuts are a temptation, and the value of sound training early in the student's career is stressed. Equipment in the laboratory should be set up and operated properly and solvents must be stored correctly. Personnel should be made aware of the flammability limits and flash points of the chemicals they use and should also be instructed about their corrosive and toxic nature and the possible effects of

exposure; permissible limits of exposure are clearly listed in tabular form in Chapter 5.

The book also discusses the adequate ventilation of fume cupboards, a factor of particular importance in the handling of isotopes of carcinogens. A separate chapter looks at the hazards from different types of radiation, special attention being given to ionizing radiation.

Sensible disposal of chemical waste is given an airing, along with the principles of good housekeeping and a format for safety inspection reports, showing safety as an integral part of the laboratory activity, not merely a set of rules on paper. Safety officers are appointed to ensure that all laboratories conform to the safety standards and that personnel are made aware of the need for safe practices in their work. In each organization, a Safety Committee representing the entire work-force should be responsible for the administration of the health and safety programme, and the authors outline the specific tasks involved in this.

At £2.75, this is a valuable text for instilling the concept of safe practice into all laboratory personnel and it should be an asset to Safety Committees. It is straightforward and uncluttered and is easy to read. While the book itself is not expensive by today's standards, the authors do point out that the cost of an adequate safety programme is high.

Cancer and the Worker. Edited by B. M. Boland and P. E. Lehman. New York Academy of Sciences, New York, 1977. pp. vi + 77. $2.00.

The recent publication of the EEC plans for health and safety in the working environment (*Off. J. Europ. Commun.* 1978, **21** (C165), 1) pointed out the need to educate individuals. Education on occupational health and safety should begin at school and continue at work—at all levels. A move towards worker awareness is being made, too, in the United States, the source of this present booklet. Much of its content is based on past and present happenings within that country, but the problems encountered have international relevance. This booklet is aimed at the worker, plant manager and union leader and begins with definitions of cancer terms, although some of the explanation remains rather 'highbrow'.

The introduction points the finger at cancer as the problem of our times. Chapters 1 and 2 openly discuss the history of cancer and provide a general description of occupational cancer in lay terms. One rather contentious statement refers to the need for Delaney-type legislation applying to cancer-causing substances in the workplace; a suggestion of dubious wisdom in the light of present problems with such legislation. However, further statements in the book concerning economic considerations for the decision-making imply that the proposed legislation need not be as restrictive as the Delaney clause.

The reader is taken on a tour of classical carcinogens, which are divided into groups (chemicals, metals, and dusts and fibres), and brief historical reviews of studies on many of the substances are included. There is a warning that exposure is not restricted to the workplace; the transport of material away from the workplace frequently occurs particularly on the worker or his clothes. DON'T SMOKE is the message in Chapter 6 to all workers and especially to those working with substances that already increase their risk of getting cancer.

Control of exposure and the adoption of suitable standards are discussed in Chapter 9 and here, too, one is led through problems already encountered with such materials as vinyl chloride. Medical tests which can be used to detect cancer in its early stages are described.

The later stages of the book take a broader look at the subject. Cancer is acknowledged as a 'social disease'—a disease whose causes are an integral part of the technology and economy of our society. Risk assessments are complex problems and although the costs of protecting workers can be high, the long-term benefits of such protection are undeniable. If the worker is to have any say in his own protection, a free movement of information is required. Hopefully this booklet will go some way to help the worker contribute ideas in a rational and informed way on the safety of his immediate environment.

Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. An Encyclopedic Handbook. Volume 3. Mycotoxicoses of Man and Plants: Mycotoxin Control and Regulatory Practices. Edited by T. D. Wyllie and L. G. Morehouse. Marcel Dekker, Inc., New York, 1978. pp. xvii + 202. Sw.fr. 140.00.

This, the final volume of an encyclopaedic handbook on mycotoxins, is concerned with man and plants. The contributors intend the whole handbook to provide the research scientist, biologist, food scientist and toxicologist with access to data in the field of mycotoxins. Detailed information is given on the identification and manipulation of mycotoxic fungi, along with methods of isolation and identification.

There is an interesting chapter in this volume on alimentary toxic aleukia, a disease that was widespread among the population of the Soviet Union several decades ago, especially in the Orenburg district, and was found to be the result of toxins produced by fungi growing in overwintered grain. The function of mycotoxins in human pulmonary disease is also discussed, although the role played by aflatoxins and other mycotoxins in diseases resulting from the inhalation of fungi is one of several aspects of the pathogenesis of fungal disease on which further study is needed.

A section of this book covers the effects of mycotoxins on higher plants, algae, fungi and bacteria, and focuses on many mycotoxins that may be harmful to plants but are not involved in the development of plant diseases. It also looks at a group of antibiotics that are possibly harmful to higher plants and animals and are usually toxic to bacteria. Concern is voiced about the problem of when and where mycotoxins get into food and feedstuffs and what can be done to prevent such contamination. Examples are given of the kinds of problems that exist and how they could be avoided. Many toxin-producing fungi have adapted to grow on harvested crops during storage, so it is important that stored products are

inspected regularly to enable signs of spoilage to be detected before mycotoxins are released.

FDA and other regulations (although not the most recent) are covered in the concluding chapter. The mycotoxin problem in the USA is considered in conjunction with the international interest in economic and public health difficulties associated with mycotoxin contamination.

The contributors to this book are informative and their articles form a useful reference text. As a whole, the volume is well presented and easy to read, the information provided being easy to locate and supported by clear diagrams and photographs.

Environmental Health Criteria 6. Principles and Methods for Evaluating the Toxicity of Chemicals. Part I. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1978. pp. 272. Sw.fr. 28.00 (available in the UK through HMSO).

This book, the first of two parts, forms a creditable and important contribution to the UNEP/WHO Environmental Health Criteria series. In contrast to earlier publications in the series, which have concentrated on evaluations of the health risks associated with specific environmental contaminants, this book is concerned with the more general problems and the methods of such evaluations. In a science that is now in such a rapid state of flux, it is refreshing to find a book that describes the current situation, gives glimpses into the future, and pin-points the areas most needing study.

For the specialist toxicologist, who may not be in close touch with some aspects of evaluation, this book will fill in the gaps. However its value is even greater for newcomers to the subject. A broad outline of chemical toxicity evaluation is adequately backed up by references for further reading. General concepts are covered in the first section and the factors that influence the design of tests are the subject of section two. Subsequent parts deal with the planning and evaluation of tests for acute, subacute and chronic toxicity, and of studies in chemobiokinetics, metabolism, morphology, inhalation exposure, and carcinogenicity and mutagenicity.

The need for a greater uniformity of methods in toxicity evaluation is recognized, and in this respect the two volumes represent further useful additions to a number of reviews and guidelines that have been produced by WHO. The second volume will cover more specialized procedures for safety evaluation and will contain chapters on functional studies of organs and systems, effects on reproduction, neurological and behavioural studies, effects on the skin and the eye, cumulation and adaptation, and factors modifying the results of toxicity testing and evaluation. It is eagerly awaited.

Biological Oxidation of Nitrogen. Edited by J. W. Gorrod. Elsevier/North-Holland Biomedical Press, Amsterdam. 1978. pp. xv + 502. Dfl. 143.00.

This monograph consists of a series of 63 papers presented at the Second International Symposium on the Biological Oxidation of Nitrogen in Organic Molecules held at Chelsea College, University of London, during September 1977. The first of these symposia on nitrogen oxidation was held in 1971 (*Cited in F.C.T.* 1973, **11**, 665). The book has been dedicated to Professor Manfred Kiese, Director of the Institute of Pharmacology in Munich, as a tribute to his pioneering work on the formation of *N*-hydroxyarylamines and their reaction with haemoglobin. Since the publication of the work of Professor Kiese and others on the production of methaemoglobinaemia in experimental animals, many other examples of *N*-oxidation reactions leading to toxic metabolites have been reported. For example, 2-acetylaminofluorene (*N*-2-fluorenylacetamide) is activated to the carcinogenic *N*-hydroxy derivative and the toxicity of certain drugs such as paracetamol (acetaminophen), iproniazid and isoniazid have been attributed to various initial *N*-oxidation reactions.

The papers presented at the meeting have been classified into seven sections and an author index is provided for the entire volume. The first two sections are devoted to the *N*-oxidation of aliphatic and aromatic amines and the *N*-oxidation of hetero-aromatic amines. Among the compounds considered are alkaloid drugs, amphetamines and related compounds, barbiturates, morpholine-containing drugs, nicotine, and nitrosamines. Unlike the carbon oxidation of drugs and other foreign compounds, where the majority of biotransformations are catalysed by the microsomal cytochrome *P*-450-dependent mixed-function oxidase complex, the enzymology of the oxidation of nitrogen in xenobiotics is more complex. In the section devoted to this topic, a paper by the Editor discusses the use of the pKa of a nitrogen-containing xenobiotic to predict whether various *N*-oxidations will be catalyzed either by cytochrome *P*-450 or by the unrelated microsomal mixed-function amine oxidase enzyme first described by D. M. Ziegler.

In studying the metabolism of nitrogen-containing xenobiotics the investigator may have to overcome practical problems in the detection, separation and quantitation of *N*-oxygenated products, as these compounds are often extremely labile, are formed only in trace amounts and can be very water-soluble. Accordingly a section for papers on analytical techniques was included in the symposium. Of particular merit here is the review paper by J. W. Gorrod and others listing both chromatographic conditions and the detection methods that have been used in studies on *N*-oxidation products. Other papers deal with various aspects of the use of the techniques of gas–liquid chromatography, high-pressure liquid chromatography, mass spectrometry and polarography.

In the section on the toxicology and pharmacology of *N*-oxidation products, two papers are concerned with the metabolism of the analgesic drug, paracetamol. A cytochrome *P*-450-dependent *N*-hydroxylation reaction has been found to be responsible for the hepatotoxic effects of this compound. Other papers in this section deal with the metabolism and subsequent toxicity of 2-acetylaminofluorene, aniline and other arylamines, cocaine, iproniazid, isoniazid, nicotine and phenacetin (acetophenetidine). The sixth section of papers considers the biosynthesis of *N*-oxidized products by plants, whilst the final section

deals with the further metabolism of *N*-oxidized products.

The papers presented at this symposium mainly contained the authors' latest research findings, only a few papers of a review type being included. The book may not appeal, therefore, to the casual reader or to those seeking basic principles, especially as the retrieval of information is greatly hampered by the absence of a subject index. However, it must be considered essential reading for those involved directly in studies on the metabolism and/or toxicity of nitrogen-containing xenobiotics.

Density Gradient Centrifugation. By R. Hinton and M. Dobrota. Laboratory Techniques in Biochemistry and Molecular Biology. Vol. 6, part 1. Edited by T. S. Work and E. Work. North-Holland Publishing Company, Amsterdam, 1976. pp. 290. $21.00.

The application of centrifugal techniques has been largely responsible for our understanding of the properties and functions of intracellular organelles. In early studies differential centrifugation was used, but this relatively simple technique is restricted in its usefulness both with respect to the purity of the fractions obtained and to the relatively large differences in particle size necessary to effect a good separation. The need to overcome these limitations has led to the increasing use of density gradient centrifugation.

The first chapter of this book defines the various types of centrifugation techniques, their use and their limitations, and covers the design, development and safety of centrifuges and rotors. Indeed, since ultracentrifuges are potentially hazardous if wrongly used or inadequately maintained, the sections on the operation of centrifuges and the care of rotors should be read by all centrifuge users. The theoretical aspects of centrifugal separations follow and in the third chapter the authors consider the choice of conditions to achieve a centrifugal separation and include discussions on rotor types, selection of density gradient solutes and the design of the gradient.

Two chapters are devoted to centrifugation in conventional (i.e. swing-out) and zonal rotors respectively. All the practical aspects of gradient preparation and loading, sample loading/unloading, centrifugation, and monitoring of the gradient are covered. The various types of zonal rotors commercially available are reviewed and the use of ancillary equipment such as pumps, gradient makers and spectrophotometers is discussed. Some of the problems and pitfalls that may be encountered in the assay of the separated fractions obtained after centrifugation are described. Another chapter is devoted to the use of density gradient centrifugation to separate the constituents of biological material, and particularly to separate organelles from tissue fractions. The authors review the approaches that have been used in studies on the subfractionation of nuclei, mitochondria, lysosomes, microsomes, chromatin and polysomes and ribosome subunits. In addition, they discuss the separation of whole cells, nucleic acids, proteins and viruses.

As with any other separation technique the results of density gradient centrifugation studies may be greatly influenced by various artefacts arising from the procedures used. The sources of such artefacts are described and include particle damage due to pelleting, high hydrostatic pressure or high solute concentration. Factors affecting the accuracy of assays of fractions from density gradients and of estimations of both particle densities and sedimentation coefficients are also discussed. The final chapter describes possible future trends in density gradient centrifugation.

In addition to the wealth of practical advice given in this book, there are four excellent appendices. These consist of a list of addresses of manufacturers of centrifuges and ancillary equipment, a glossary of terms, a list of densities and sedimentation coefficients of rat liver-cell organelles and the theory of preparation of density gradients. The text is amply illustrated and cites over 330 references.

This book provides the reader with practical advice both on the experimental approaches involved in density gradient centrifugation studies and on the interpretation of the results obtained. It is recommended as essential reading to anyone who wishes to use this technique.

BOOKS RECEIVED FOR REVIEW

Side Effects of Drugs Annual 3. A worldwide yearly survey of new data and trends. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1979. pp. xvi + 469. Dfl. 120.00.

Toxic Substances Sourcebook. Edited by S. S. Ross. Environment Information Center, Inc., New York. 1978. pp. 554. $76.00.

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

GLC and HPLC Determination of Therapeutic Agents. Part 2. Edited by K. Tsuji. Marcel Dekker, Inc., New York, 1978. pp. xiv + 520. Sw.fr. 95.00.

Cancer Registration and its Techniques. Edited by R. MacLennan, C. Muir, R. Steinitz & A. Winkler. IARC Scientific Publications no. 21. International Agency for Research on Cancer, Lyon, 1978. pp. xii + 235. Sw.fr. 40.00 (available in the UK through HMSO).

Handbook of Clinical Neurology. Vol. 36. Intoxications of the Nervous System, Part I. Edited by P. J. Vinken and G. W. Bruyn. Elsevier/North-Holland Biomedical Press, Amsterdam, 1979. pp. xii + 570. Dfl. 240.00.

Hepatotoxicity. The Adverse Effects of Drugs and other Chemicals on the Liver. By H. J. Zimmerman. Prentice-Hall International, London, 1978. pp. x + 597. £35.40.

Potential Industrial Carcinogens and Mutagens. Studies in Environmental Science 4. By L. Fishbein. Elsevier Scientific Publishing Company, Amsterdam, 1979. pp. x + 534. Dfl. 150.00.

Environmental Health Criteria. 7. Photochemical Oxidants. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1979. pp. 110. Sw.fr. 10.00 (available in the UK through HMSO).

Toxicity of Heavy Metals in the Environment. Part 2. Edited by F. W. Oehme. Marcel Dekker Inc., New York, 1979. pp. x + 970. $45.00.

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Pergamon Press Ltd 1979. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

MSG—FOOD FOR THOUGHT

Monosodium glutamate (MSG) has been suspected of causing brain damage in neonates of various species (*Cited in F.C.T.* 1977, **15**, 347), and hypothalamic damage resulting in multiple endocrine dysfunction in adult mice that had been treated neonatally with MSG has also been reported (*ibid* 1978, **16**, 393). However, factors such as species, age, route of administration and experimental design have introduced wide variations into the results of experiments (*ibid* 1977, **15**, 348) and the dietary threshold level at which MSG exerts neurotoxicity has yet to be established (*ibid* 1979, **17**, 83). In the following studies, the effects of orally administered MSG on various species are described.

Oral administration of 1 g MSG/kg body weight in a 10% (w/v) solution produced similar overall plasma levels of glutamic acid (GA) in adult mice and rats. Peak plasma levels and apparent half-lives of GA were also similar in both species. However, when the same dose was administered to adult guinea-pigs, the overall plasma levels and the apparent half-lives were higher (Bizzi *et al. Toxicology Lett.* 1977, **1**, 123). After a dose of 1 g MSG/kg in 10% (w/v) solution, the overall plasma levels were higher in neonatal (7-day-old) mice and rats than in the adults, but the reverse was true for guinea-pigs. The GA levels in mouse and guinea-pig brains were not altered by orally administered MSG until the peak plasma-GA levels exceeded the basal concentrations by about 20 times.

The concentration at which MSG was administered influenced plasma-GA levels. When a dose of MSG was administered to 7-day-old animals (mice: 0·5 g/kg; rats: 1·0 g/kg) in solutions of differing concentration (mice: 2–20%, w/v; rats: 2–10%, w/v), the peak plasma-GA levels and overall plasma levels, but not the apparent half-life of GA, increased with increasing concentration (Bizzi *et al. loc. cit.*). Similar effects occurred in adult animals. Overall and peak plasma levels also increased with increasing doses of MSG given at the same concentration. The effect of concentration on plasma-GA levels was observed also in human volunteers, in whom 60 mg MSG/kg produced higher peak and overall plasma levels when given at 8% (w/v) than at 2% (Bizzi *et al. loc. cit.*).

Contradictory evidence comes, though, from a study in mice by James *et al.* (*Toxicology Lett.* 1978, **1**, 195). In 43-day-old male mice given a single oral dose of 1·5 g MSG/kg at various concentrations (2–20%) in aqueous solution, plasma-GA levels were increased at all concentrations given, the highest values being attained 15 min after dosing. However, the increase in GA level was not related to the concentration of the solution used, although the sodium concentration in the plasma did vary with the concentration of the MSG solution. Between 15 and 90 min after dosing, significantly lower plasma-sodium concentrations were produced by the 2% MSG solution than by the other concentrations. At 15 min, the 5% MSG solution produced lower plasma-sodium concentrations than 10 or 20% MSG, and at 90 min, 20% MSG produced a higher plasma-sodium concentration than the lower strengths. After 120 min, there was no difference, however, and the values for sodium concentration were within the normally accepted range at all times. Although the plasma-GA levels were increased by dosing with MSG, no histological damage was induced in the hypothalamus, and it is assumed that the blood–brain barrier in adult mice protects against such damage.

It has been suggested that considerable amounts of MSG appear to be harmless when ingested as part of an otherwise normal diet (*Cited in F.C.T.* 1975, **13**, 125). Further support for this view comes from studies on beagle dogs (Owen *et al. Toxicology Lett.* 1978, **1**, 217) and rats (*idem, ibid* 1978, **1**, 221). Beagle dogs were fed Purina Dog Chow containing either 5·13% (w/w) sodium propionate or 2·5, 5·0 or 10·0% (w/w) MSG for 104 wk. No adverse effects were observed on weight gain, food consumption, efficiency of food conversion, or behaviour; nor were any differences noted between the electrocardiograms, ophthalmological findings, haematology and serum chemistry, organ weights or mortality of experimental and control dogs. Both the dietary additives slightly increased urine volume and sodium excretion, but urinary concentration ability was not impaired. Tissue samples, including kidney, taken after treatment for 13 or 104 wk showed no histopathological changes attributable to the additives. The medullary tubular lumen in both treated and control dogs showed foci of mineralization. Similarly, in rats fed 2·05% sodium propionate or 1·0, 2·0 or 4·0% MSG in the diet for 104 wk there were no adverse effects on weight gain (except for a slight lowering in males given 4·0% MSG or 2·05% sodium propionate after wk 60), or on food consumption, efficiency of food conversion, blood, organ weights or mortality (with the exception of females receiving 1% MSG, who survived better than the controls). The highest feed levels of MSG and sodium propionate increased water consumption, urine volume and sodium excretion; an increased incidence and earlier onset of spontaneous subepithelial basophilic deposits in the renal pelvis may have been related to the increased urine volume and sodium excretion but were not thought to be specifically associated with MSG. As in dogs, focal mineralization in the medullary tubules was equally incident in treated rats and in controls.

O'Hara *et al.* (*J. toxicol. Sci., Japan* 1977, **2**, 281) investigated the effects of the route of MSG adminis-

tration on plasma-GA levels in mice. A single dose of 1 g MSG/kg in 10% (w/v) aqueous solution was given ip, sc or by gastric intubation to mice aged 10 days, 23 days or 4 months. Plasma-GA levels reached a peak 10–30 min after the dose was given and returned to basal values after 90 min. After oral doses, the GA peaks were lower than after ip or sc injection, particularly in the 10-day-old and 23-day-old animals.

Single doses of 1 g MSG/kg, mixed into human-infant formula feed or clear soup to give concentrations of 10% (w/v), were administered, respectively, to 10-day-old or adult mice by gastric intubation. The peak plasma-GA levels were reduced by some 50% in mice given the dose in either of the two foods, compared with the levels in mice given the same dose in an aqueous solution. When 1 g MSG/kg was administered to 23-day-old and adult mice as part of a normal diet, only slight elevations in plasma-GA levels occurred and peak values were less than 45 μmol/100 ml compared with 219 and 344 μmol/100 ml in 23-day-old and 4-month-old mice, respectively, given the dose by gastric intubation in aqueous solution.

Other studies in rats (*Cited in F.C.T.* 1971, **9**, 719) and in neonatal pigs (*ibid* 1975, **13**, 125), however, did not show any reduction in peak GA levels in the plasma when MSG was administered with food rather than alone in solution. Nevertheless, the rates of increase to the peak level were slower, and on balance these studies do suggest that the effects of MSG may be influenced to a significant degree by accompanying feeding patterns or by other dietary components.

[P. Cooper—BIBRA]

VINYL CHLORIDE—PART 2: MUTAGENICITY

In the last issue (p. 403), we reviewed the more important metabolic studies on vinyl chloride (VC). Now we turn to mutagenicity. This aspect of VC's biological profile has been extensively investigated, particularly in bacteria.

Mutagenicity in Salmonella typhimurium

The preliminary studies of mutagenicity involved the Ames test. When VC, at a concentration of 20% (v/v) in air, was incubated with *Salmonella typhimurium* strain TA1535 and a mammalian metabolizing system, namely a fortified post-mitochondrial rat-liver supernatant, the number of histidine revertants was increased to three times the spontaneous mutation rate (Rannug *et al. AMBIO* 1974, **3**, 194). An increase over the spontaneous mutation rate also occurred in strains TA1535, TA1530 and G46 when they were exposed to a similar VC atmosphere in the presence of a 9000-**g** mouse-liver supernatant (S-9 fraction) fortified with an NADPH-generating system (Bartsch *et al. Int. J. Cancer* 1975, **15**, 429). The greatest response was seen with TA1530, VC treatment (for 48 hr) being associated in this case with a 28-fold increase in the number of histidine revertants. The VC concentration of the incubation medium below the 20% atmosphere was found to be 0·004 M at equilibrium. Although exposure to 20% VC in air proved to be mutagenic towards TA1530 in the absence of metabolic activation, mutagenic activity was doubled by the addition of liver fractions from phenobarbital-treated mice. The highest mutagenic responses occurred in the presence of a 9000-**g** supernatant or with the recombined microsomal and soluble protein fraction in the presence of an NADPH-generating system. Purified microsomal fractions were less efficient activators, and soluble liver proteins (100,000-**g** supernatant) alone were almost without effect. The addition of alcohol dehydrogenase and NAD^+ to either the fortified post-mitochondrial or the 100,000-**g** liver supernatant did not increase the mutation rate (Bartsch *et al. loc. cit.*).

In the TA1535 and TA100 strains, McCann *et al.* (*Proc. natn. Acad. Sci. U.S.A.* 1975, **72**, 3190) found that although a VC atmosphere (20% v/v) gave evidence of direct mutagenic activity, its activity was doubled by the addition of the S-9 fraction from a PCB-treated rat. The results of a similar study by Andrews *et al.* (*Mutation Res.* 1976, **40**, 273) seem to be anomalous, in that VC at levels of up to 15% in air were shown to be directly mutagenic in TA1535 and an S-9 fraction from PCB-treated rats produced only a slight enhancement. However, the differences between these two sets of results may partly be explained by the different incubation times involved.

Vinyl chloride is not thought to be mutagenic *per se*. The increase in the number of histidine revertants seen when VC is incubated with strains TA1530, TA1535 or TA100 in the absence of any mammalian metabolizing system is thought to be a result of the non-enzymic breakdown products of VC or of compounds formed by the bacterial enzyme system (Bartsch *et al. loc. cit.*).

Aqueous solutions of VC (initial concentration 0·083 M) gave no evidence of mutagenicity when incubated with *S. typhimurium* strains TA1530, TA1535 or G46 together with a fortified 9000-**g** liver supernatant from phenobarbital-treated mice (Bartsch *et al. loc. cit.*). A similar lack of activity of VC in solution (at an initial concentration of 0·022 M) was reported by Elmore *et al.* (*Biochim. biophys. Acta* 1976, **442**, 405) in TA100. It was thought that the inactivity of VC in these systems may have been caused by rapid diffusion of monomer from the liquid phase into the atmosphere.

S. typhimurium strains TA1530, TA1535, TA100 and G46 respond with varying sensitivity to monofunctional alkylating agents (base-pair substitutions or deletions). Strains TA1536, TA1537 and TA1538, which are specifically reverted by frameshift mutagens, were unaffected by 20% VC atmospheres even in the presence of liver fractions from rats or mice (Bartsch *et al. loc. cit.*; Rannug *et al. loc. cit.*).

The effects of the various liver fractions seen in the studies reviewed by Bartsch & Montesano (*Mutation Res.* 1975, **32,** 93) indicate that the mixed-function oxidases are responsible for the conversion of VC to mutagenic metabolites in mammals. Garro and his colleagues (*ibid* 1976, **38,** 81), however, did not believe that the hepatic fractions were exerting their effect by an enzymatic mechanism. In contrast to the results of Bartsch *et al.* (*loc. cit.*) who reported that 20% VC in air induced a mutagenic response in TA1530 only when an NADPH-generating system was present, Garro *et al.* (*loc. cit.*), testing 75% VC in air in the same Salmonella strain, found that the activating effect of the S-9 microsomal fraction on VC mutagenesis was independent of the NADPH-generating system (NADPH being a necessary co-factor for the mixed-function oxidases) and was largely unaffected by a thermal treatment that would be sufficient to inactivate all enzyme activity. The increases in VC-induced mutagenicity observed when the liver fractions were obtained from either untreated or PCB-treated animals were similar, although the PCB would be expected to increase the mixed-function oxidase content of the liver. Garro *et al.* (*loc. cit.*) considered, therefore, that the liver fraction might have been increasing the mutagenicity of VC non-enzymatically, perhaps by promoting the formation of free radicals. A free-radical generating system, riboflavin irradiated with UV light, doubled the number of VC-induced revertants in the *S. typhimurium* (TA1530) system, and this stimulation was further enhanced by the presence of a photopolymerization accelerator—*N,N,N',N'*-tetramethylethylenediamine.

Mutagenicity of VC metabolites

The two suspected VC metabolites, chloroethylene oxide and 2-chloroacetaldehyde each directly increased the number of histidine revertants in strain TA1530 of *S. typhimurium.* At initial concentrations of 0·4 μmol/ml the oxide was about twice as active as the aldehyde with respect to mutagenicity, but chloroethylene oxide exhibited a lower toxicity; 11% of a cell population survived an oxide concentration of 0·4 μmol/ml while less than 0·004% of the Salmonella cells exposed to the same concentration of the aldehyde survived (Malaveille *et al. Biochem. biophys. Res. Commun.* 1975, **63,** 363).

Chloroethylene oxide was strongly mutagenic towards TA1535. At an initial concentration of 0·45 mM, the spontaneous mutation rate of three mutants/10^8 cells was increased to 96/10^8 surviving cells. Chloroacetaldehyde at an initial concentration of 0·5 mM only increased the spontaneous mutation rate approximately three-fold (Rannug *et al. Chemico-Biol. Interactions* 1976, **12,** 251). It was calculated that chloroethylene oxide, with a half-life in solution of only a few minutes at 37°C, was some 450 times as mutagenic as the aldehyde on a mol:mol basis. The oxide (tested at 0·26 M) and 2-chloroacetaldehyde (0·1 M) were also shown to be mutagenic in Salmonella strain TA100 (Elmore *et al. loc. cit.*). Because of its instability, an increased number of histidine revertants were observed when the oxide was pre-incubated with the test micro-organism at 3°C. All of the four possible forms of 2-chloroacetaldehyde exhibited a mutagenic response against TA100, the pure compound was more active than the monomer hydrate which in turn was more active than the dimer hydrate and trimer, probably demonstrating the importance of the intact electrophilic carboxyl group in eliciting a mutagenic response (Elmore *et al. loc. cit.*).

In the presence of a post-mitochondrial mouse-liver fraction, 2-chloroethanol (108 μmol/plate) increased the number of histidine revertants in the TA1530 strain of *S. typhimurium* about ten times; distinct mutagenic activity, but of a lower order, was also observed in the absence of metabolic activation (Bartsch *et al. loc. cit.*). Chloroacetic acid induced neither a direct nor a tissue-mediated response in this strain. In the study by McCann *et al.* (*loc. cit.*) 2-chloroethanol was weakly mutagenic against TA100, inducing 0·6 revertant colonies/μmol, and demonstrated a trace of activity in TA1535. Microsomes significantly increased the number of compound-induced histidine revertants in TA100 whilst horse-liver alcohol dehydrogenase together with NADH had no analogous effect. Elmore *et al.* (*loc. cit.*) observed no mutagenic response when 2-chloroethanol was incubated at 1 mM with strain TA100. When tested by Rannug *et al.* (1976, *loc. cit.*) 2-chloroethanol and chloroacetic acid were without effect on TA1535 at concentrations up to 1.5 mM much higher concentrations of 2-chloroethanol (1 M) proved to be weakly mutagenic.

Mutagenicity in other systems

In *Bacillus subtilis,* chloroethylene oxide and 2-chloroacetaldehyde selectively inhibited strain MC-1, a mutant lacking recombination repair of DNA; chloroethanol, chloroacetic acid and VC itself (in solution) were inactive in this system (Elmore *et al. loc. cit.*). Chloroethylene oxide, at concentrations of up to 25 μM, and 2-chloroacetaldehyde, at concentrations up to 12·8 μM, also produced a dose-dependent induction of 8-azaguanine- or ouabain-resistant mutants in Chinese hamster V79-4 cells *in vitro,* but 2-chloroethanol or chloroacetic acid were inactive at concentrations nearly 100 times higher (Huberman *et al. Int. J. Cancer* 1975, **16,** 639).

In the presence of mouse-liver microsomes, vinyl chloride atmospheres (up to 50% v/v) significantly increased the forward mutation frequency of *Schizosaccharomyces pombe* and induced gene conversion in *Saccharomyces cerevisiae* (Loprieno *et al. Mutation Res.* 1976, **40,** 85). No mutagenic activity was apparent in the absence of the microsomal fraction. Chloroethylene oxide was directly and highly mutagenic in these test systems (*idem, Cancer Res.* 1977, **36,** 253). In either the presence or absence of microsomes, a commercial aqueous solution of 2-chloroacetaldehyde exhibited only feeble genetic activity, whilst 2-chloroethanol was totally inactive. Since a concentration of 0·05 mM chloroethylene oxide induced the same mutagenic frequency as 50 mM VC, it seems likely that all the activity of the monomer in this system could be ascribed to the oxide.

In a host-mediated assay in the mouse, VC given at 700 mg/kg by gavage significantly increased the mutation frequency of *Sch. pombe,* which had been injected into the peritoneal cavity during 12 hr of treatment (Loprieno *et al.* 1976, *loc. cit.*). Methyl methanesulphonate, used as a positive control, proved

to be almost 200 times as active as VC. No significant increases in forward mutation were seen 3 or 6 hr after an ip injection of 250 mg 2-chloroacetaldehyde/kg (Loprieno *et al.* 1977, *loc. cit.*).

Either in ethanolic solution or in the gaseous state, VC induced no detectable mutagenic change in two strains of *Neurospora crassa*, a eukaryotic microorganism (Drozdowicz & Huang, *Mutation Res.* 1977, **48**, 43). The addition of an S-9 liver fraction from either control or phenobarbital-treated rats had no effect. The apparent lack of sensitivity of this system may have been due to the inability of VC and its metabolites to penetrate the conidia.

A significant increase in the frequency of recessive lethals over that in controls was observed in male *Drosophila melanogaster* exposed to VC at 850 ppm for 2 days and there was a slight increase in mutation rate on exposure to 30 ppm for 17 days (Verburgt & Vogel, *ibid* 1977, **48**, 327). No further enhancement in the recessive lethal incidence was seen after 2-day exposures to VC levels in excess of 10,000 ppm. It was suggested that above this concentration the Drosophila enzymes were no longer capable of metabolizing (activating) additional VC. In contrast to the recessive lethal assay, negative results were obtained in tests on dominant lethals, translocations and entire and partial sex-chromosome loss in Drosophila exposed to VC at 30,000 ppm for 2 days (Verburgt & Vogel, *loc. cit.*).

VC has failed to produce dominant lethals when tested in a mammalian system. Male CD-1 mice, exposed to 3000, 10,000 or 30,000 ppm VC for 6 hr/day for 5 days, were housed for 5 days in each of the following 8 wk with successive pairs of untreated females (Anderson *et al. Mutation Res.* 1976, **40**, 359). Using the female mice as the indicator organism there was no evidence that VC had any mutagenic effects on any maturation stage of spermatogenesis. There was no significant increase in the number of post-implantation early foetal deaths, no evidence of pre-implantation egg loss (except at the highest VC dose, where the loss showed a low level of significance but could largely be attributed to a single female), nor any reduction in fertility. A dominant lethal effect was clearly demonstrated in the positive controls given a single ip injection of 200 mg cyclophosphamide/kg or five oral doses of 200 mg ethyl methanesulphonate/kg.

In most of the Salmonella studies, VC demonstrated activity only in the presence of a microsomal liver fraction. The Ames test has given conflicting evidence on metabolism; whilst the studies of Bartsch *et al.* (*loc. cit.*), in line with the other *in vitro* studies, suggested that the microsomal fraction of the liver was acting enzymatically, the experiments of Garro *et al.* (*loc. cit.*) indicated, so far uniquely, that a free-radical mechanism was responsible for the conversion of VC to its active metabolites. VC elicited a mutagenic response in a simple eukaryotic system, demonstrating activity in *Sch. prombe* and *Saccharomyces cerevisiae* both *in vitro* in the presence of liver microsomes and in a host-mediated assay. The incidence of recessive lethal mutation was increased in Drosophila treated with 30 ppm VC for 17 days. Chloroethylene oxide and 2-chloroacetaldehyde have proved to be mutagens—the oxide particularly so—in prokaryotic micro-organisms, namely the strains of Salmonella susceptible to frameshift mutagens and *B. subtilis*. This evidence further implicates chloroethylene oxide as the active VC metabolite.

No evidence of activity was seen in the dominant lethal assay in mice. However, further investigations of mutagenicity in mammals are required before an assessment of the genetic hazards presented by VC to man will be possible.

[J. Hopkins—BIBRA]

CADMIUM AND THE TESTIS

The widespread distribution of cadmium (Cd) in the environment, and its multifaceted toxicity, have prompted innumerable studies. Cd has a tendency to accumulate in the mammalian kidney (*Cited in F.C.T.* 1975, **13**, 470), is suspected of contributing to hypertension (*ibid* 1977, **15**, 480), and has been implicated in some cases of impaired respiratory function (*ibid* 1977, **15**, 479) and in the Japanese condition known as 'itai-itai' (*ibid* 1977, **15**, 478). The mammalian testis is highly vulnerable to Cd, which has a primary effect on the testicular vasculature and which possibly exerts a secondary action by modifying the effect of endogenous testosterone (*ibid* 1970, **8**, 317; *ibid* 1971, **9**, 592).

In a recent paper describing the early changes in the microvascular bed of the testis of rats injected ip with $CdCl_2$, Aoki & Hoffer (*Biol. Reprod.* 1978, **18**, 579) have come out strongly in support of the view that the testicular changes seen following Cd treatment are secondary to ischaemia rather than direct effects of the Cd. More details about the early changes in the vascular supply of the testes of the Cd-treated rat were derived from an electron-microscopic study designed specifically to demonstrate possible differences between cryptorchid and scrotal testes (Fende & Niewenhuis, *ibid* 1977, **16**, 298). The testes of rats given a single sc injection of 5 mg $CdCl_2$/kg showed mild oedema after 3 hr; after 6 hr this had increased, and at 24 hr the interstitial spaces showed massive haemorrhage and oedema, and the tubules showed degenerative changes. These effects were more extensive in the cryptorchid than in the scrotal organs. The ultrastructural alterations consisted of changes in the endothelial-cell junctions of the intra-testicular capillaries, and progressed from a decrease in membrane-associated electron density at 3 hr to separation of the endothelial cells 6 hr after the injection.

The latest round of studies on the morphological effects of Cd on the testis also included a paper by Donnelly & Monty (*Toxicology Lett.* 1977, **1**, 53), who studied the toxicity of Cd administered by various routes to dogs. Single iv doses of 4 or 8 mg $CdCl_2$/kg were lethal within 48 and 24 hr respectively, causing convulsions with uncontrollable vomiting and defae-

cation. After dosing at either level, haemorrhagic and necrotic foci appeared in the testes. The same dose given sc produced severe inflammation, oedema, haemorrhage and necrosis at the injection site, but no histological changes in the testis, and when injected intramuscularly, it produced severe local inflammation, but only a few haemorrhagic and necrotic foci in the testes. Intra-testicular infusion of 0·1 mg $CdCl_2$/kg produced necrosis and haemorrhage into the central portion of the testis, but the peripheral tubules remained normal. At twice this dose, intra-testicular infusion caused massive testicular necrosis, particularly in the central part, and shrinking and disorganization of most peripheral tubules, with extensive degeneration of Leydig cells, which lie in the interstitial tissue between the tubules and are associated with testosterone production. Initial atrophy of the seminiferous tubules was followed by interstitial revascularization and regeneration of Leydig cells. However, some disorganization persisted, with signs of limited regeneration and spermatogenesis, 24 wk after the injection. Epididymal damage had subsided by wk 24, but ejaculate from the treated dogs remained aspermic.

In golden hamsters, single sc injections of 9·12 mg $CdCl_2$ caused damage to the seminal epithelium, Sertoli cells and Leydig cells of the testis (Girod & Dubois, *Annls Endocr.* 1976, **37**, 273). The effect was marked after day 4, but by wk 7 the interstitial tissue was beginning to regenerate, although the tubular lesions persisted throughout the 6-month observation period. Another effect of this treatment was seen in the anterior lobe of the pituitary, in which gonadotropic cells became hyperplastic, while corticotropic cells showed no detectable response.

In rats, a single sc injection of 1 or 5 mg $CdCl_2$ (approximately 2·5 or 12·5 mg/kg) reduced significantly the weight of testes, epididymis, vas deferens, ventral prostate and seminal vesicles, and caused a severe reduction of the sperm count in the vas deferens, caput and cauda epididymidis (Saksena *et al. Biol. Reprod.* 1977, **16**, 609). No change in libido or fertility was apparent 7 or 15 days after the lower dose, but both were suppressed in animals given 5 mg $CdCl_2$. Both doses reduced serum levels of testosterone and 5α-dihydrotestosterone, but not androstenedione. This suggests that the testis failed to synthesize testosterone, not because of a shortage of the precursor (androstenedione) but because the necessary enzyme system was impaired. The authors postulate, on the basis of earlier work, that thiol-group inhibition by Cd might be involved in this impairment.

Further evidence that $CdCl_2$ treatment not only affects spermatogenesis but also suppresses androgen production was obtained from a study in hamsters carried out by the same group (Lau *et al. ibid* 1978, **19**, 886). In this case, a dose of 1 mg $CdCl_2$ (about 7 mg/kg), given sc, reduced the serum and testicular concentrations of androstenedione and progesterone as well as of testosterone and 5α-dihydrotestosterone. The effect was most marked, however, in relation to testosterone.

The findings of Bordás *et al.* (*Arch. Tox.* 1976, **36**, 163) were consistent with the hypothesis that Cd acts by inactivating thiol groups. These authors reported that daily dietary administration of 50 mg L-cysteine (an essential sulphur-containing amino acid which can be produced from methionine in the liver) protected the rat testis from the damaging effects of four weekly sc doses of 1·0 mg $CdCl_2$/kg. On the other hand, DL-ethionine (an amino acid antagonist which blocks the hepatic conversion of methionine to cysteine) enhanced the testicular damage induced by this sc dose of $CdCl_2$, when it was given as a dietary supplement providing an intake of 10 mg/rat/day.

Another approach to the mechanism of Cd toxicity in the testis has focused on the recently demonstrated role of polyamines in cell metabolism, and particularly in nucleic acid metabolism. Furata (*Toxicology Lett.* 1977, **1**, 141) gave rats a single sc injection of 5–50 μmol $CdCl_2$/kg (approximately 1–9 mg/kg) and at intervals of between 3 and 72 hr examined the testes histologically and determined their polyamine (spermine and spermidine) content. Histopathological changes were observed only with doses above 10 μmol/kg (about 2 mg/kg). Spermidine, but not spermine, was decreased at 48 hr by 5–10 μmol/kg, and both polyamines were decreased by injection of more than 15 μmol $CdCl_2$/kg; a dose of 25 μmol/kg (4·5 mg/kg) reduced the level of spermidine to about 27% of the control value at 24 hr. The maximal reduction in testicular spermidine preceded that of spermine by about 24 hr, a finding in accord with the known metabolic conversion of the former compound to the latter. Administration of a total of 2·5 mmol zinc acetate/kg in three doses given before, with and after a dose of 25 μmol $CdCl_2$/kg not only protected the rat testis from histological damage but prevented the Cd-induced reduction in polyamine levels. The known ability of Zn to afford protection against the induction of testicular lesions is thus effective also against the effects of Cd on testicular polyamines. Furata (*loc. cit.*) also points out that changes in spermidine levels appear to be a more sensitive indicator of Cd toxicity than are histological changes.

In connection with their study on the early changes induced by Cd in the microvasculature of the rat testis, Aoki & Hoffer (*loc. cit.*) speculated on the possible involvement of a specific testicular carbonic-anhydrase isoenzyme, which has been shown to be sensitive to Cd (Hodgen *et al. Biol. Reprod.* 1970, **2**, 197). Histochemical studies have demonstrated carbonic anhydrase activity in the testis in locations coinciding with the endothelium of the capillaries (Cohen *et al. ibid* 1976, **14**, 339), and more work on this aspect is in hand. The possible involvement of carbonic anhydrase in the mechanism of Cd toxicity in the testis is not a new idea. The known capacity of zinc to counteract the toxic effects of Cd, the fact that carbonic anhydrase is a zinc-containing metalloenzyme, and the transient decrease in its activity detected in the testes after Cd administration (Hodgen *et al. J. Reprod. Fert.* 1969, **18**, 156; Johnson & Walker, *ibid* 1970, **23**, 463) combined to suggest an inhibition of carbonic anhydrase activity resulting from Cd-zinc competition in the enzyme structure as the cause of Cd-induced testicular necrosis.

The work of Alsen *et al.* (*Arch. Tox.* 1976, **37**, 39), however, has provided no support for this view. These authors gave rats a single ip injection of $CdCl_2$ equivalent to 1·5, 3 or 5 mg Cd ion/kg and determined carbonic anhydrase activity and haemoglobin levels

in the blood and testes after 0·25–48 hr. Studies were also carried out on testes perfused via the testicular artery. By these means it was possible to assess how much of the carbonic anhydrase activity in the testis was contributed by the erythrocytes in the circulating blood and how much was derived from the local tissues. From the findings, it was concluded that alterations in the carbonic anhydrase activity of the testes following Cd administration reflected changes in the blood content and therefore in erythrocyte carbonic anhydrase in the organ.

The exact role played by these and possibly other factors in the damaging effects exerted on the testis by Cd remains to be established. The problem continues to attract wide interest, however, and it may be that the placing of a few more key pieces will enable the rest of the jigsaw to be fitted into place.

[P. Cooper—BIBRA]

CADMIUM AND THE PROSTATE

In addition to the affinity of cadmium (Cd) for the testis (see above), there is considerable evidence linking occupational exposure to Cd with diseases of the prostate, including prostatic carcinoma (Khare *et al. Res. Commun. chem. Path. Pharmac.* 1978, **20**, 351; Kipling & Waterhouse, *Lancet* 1967, **I**, 730; Potts, *Ann. occup. Hyg.* 1965, **8**, 55). The lateral prostate of the rat contains high levels of zinc, the uptake of which is regulated by testosterone and affected by Cd (Timms *et al. Virchows Arch. Abt. B Zellpath.* 1977, **25**, 33) and direct injection of Cd into the rat prostate has been shown to induce local carcinoma (Aughey *et al. Br. J. Urol.* 1975, **47**, 185). Whether the action of Cd on the prostate is principally a direct effect or an indirect result of the lowering of testosterone levels has been in some doubt, however.

A study designed by Timms *et al.* (*loc. cit.*) to demonstrate whether direct ultrastructural changes could be demonstrated in the prostate of Cd-treated rats prior to the lowering of testosterone levels has indicated that Cd does exert some direct effect on this organ as well as on androgen levels. The ultrastructure of the lateral prostate of the rat after a single sc dose of 0·02 mmol $CdCl_2$/kg body weight was examined at intervals of 1–20 days. Zinc and Cd in tissues were estimated by atomic absorption spectroscopy. The zinc content of the prostate declined after this administration of Cd, although that of the testes did not alter appreciably. The dry-weight content of Cd was tenfold higher in the prostate than in the testis, and continued to increase for the first 10 days. Plasma testosterone fell to 20% of its initial value within 2 days of Cd injection, and had dropped below 10% after 20 days. Plasma luteinizing hormone, having decreased slightly during the first 2 days, was above control values at 5 and 10 days, whereas plasma prolactin did not alter significantly. Ultrastructural changes in lateral prostate epithelium at 24 hr showed dilatation of the endoplasmic reticulum, a decreased number of microvilli, and a virtual absence of secretory granules. The membrane of the basal cells adjacent to the stroma showed increased pinocytotic activity. After 2 days most of the characteristics of the control organs were present but there was less secretory activity, the mitochondria were elongated, with a dense matrix, and were confined mainly to apical and basal cell regions. After 5 days many prostatic cells showed concentric whorls of rough endoplasmic reticulum in the Golgi region, with small dense mitochondria predominating in the upper and lower cell extremities. Changes after 10 days were similar but more marked. The most prominent change at 20 days was to relatively undifferentiated epithelial cells containing a large nucleus.

Electron microscope microanalysis showed that the presence of Cd in subcellular organelles of the lateral epithelium was associated with the dilatation of the endoplasmic reticulum, loss of secretory granules and reduction in Golgi activity. After 1 day, Cd was demonstrated in the nucleoli and lysosomes of the epithelial cells and in the secretory material in the lumen; after 20 days it was detected only in the nuclei of (new) epithelial cells. Involutionary changes observed at 2, 5 and 10 days were considered to be the result of decreased testosterone levels following Cd-induced testicular injury. Overall, this study indicated that the marked ultrastructural changes in the rat lateral prostate correlated with the presence of Cd and were exacerbated by the ensuing hormonal imbalance.

In a related *in vitro* study, lateral prostate explants from rats were cultured for 6 days with or without added testosterone and/or 10 or 100 mM-$CdCl_2$ (Chandler & Timms, *Virchows Arch. Abt. B Zellpath.* 1977, **25**, 17). Normal epithelium was not maintained in any of these cultures. The epithelial cells became necrotic and were replaced by basal cells which showed varying degrees of differentiation according to the culture conditions. Although incomplete, differentiation was most advanced in the testosterone-supplemented cultures; the newly growing cells were cuboid in shape and developed microvilli similar to those in normal tissues but were poorly supplied with endoplasmic reticulum and secretory granules. Cd had a direct effect on the cells, particularly at the higher level, causing necrosis of the basal cells as well as of the original epithelial cells. Differentiation was markedly impaired in the Cd cultures even in the presence of testosterone, although apical microvilli were still observed with the lower Cd level. Cd was shown to be incorporated in both the epithelial and basal cells, becoming located in the nucleus and within cytoplasmic organelles.

In both this and the *in vivo* study (Timms *et al. loc. cit.*) investigation of the subcellular distribution of zinc and Cd provided evidence in support of the view that these two elements compete for binding sites within the cells.

Khare *et al.* (*Res. Commun. chem. Path. Pharmac.* 1978, **20**, 351) compared the effects of injecting Cd

or another heavy metal, lead, directly into the prostate of rats. The lead injection (1 mg lead acetate/rat) caused calcification of both bladder and prostate, stromal oedema and excessive periprostatic fibrosis, accompanied in a few cases by stone formation in the bladder. The 1-mg dose of $CdCl_2$, on the other hand, reduced the size and weight of the prostate, and caused marked atrophy of the gland, with formation of cuboidal epithelium and squamous metaplasia in the prostatic acini. While the latter may suggest a progressive precancerous change, no tumour formation occurred in these rats, which were, however, only kept alive for 60 days after treatment. The testes in these Cd-treated rats were small and characterized by almost complete degeneration of the seminiferous tubules, peritubular and interstitial fibrosis and calcification of the tubules. There was no evidence of any synergistic effect when lead acetate (0·5 mg) and $CdCl_2$ (0·5 mg) were injected together into the rat prostate.

As a postscript to these comments on the effects of Cd on the male reproductive system, it is perhaps appropriate to draw attention to the female rat's relative resistance to this element. Der *et al.* (*ibid* 1976 **14**, 689) found that repeated daily intramuscular injections of 250 μg Cd (as $CdCl_2$) to male rats reduced the size of prostate, testes and epididymis, caused ulcers at the injection site and generally caused more severe reactions and poorer health than occurred in females given similar injections for 54 days. The latter showed some decrease in uterus, ovary and pituitary weights, and in both sexes the liver, spleen and kidneys were enlarged. Males are reported to accumulate more Cd than females under similar conditions of exposure, possibly because of the greater demethylating potential of the female liver.

[P. Cooper—BIBRA]

MORE LIGHT ON PYRROLIZIDINE ALKALOIDS

The natural occurrence and toxic properties of pyrrolizidine alkaloids have been reviewed extensively before (Cooper, *Fd Cosmet. Toxicol.* 1974, **12**, 559) and a great deal more information is gradually becoming available. Most of the new information concerns the alkaloids of *Senecio jacobaea*, the ragwort, and to a lesser extent *S. vulgaris*, the common groundsel.

Effects on rodents

Significant changes in the histology of the livers, kidneys and lungs of mice have been reported after the feeding of diets containing dried *S. jacobaea* (Hooper, *J. Path.* 1974, **113**, 227). The powdered plant was fed at a dietary level of 10% for 9 wk and then at 20% for the rest of the experimental period of 193 days. The dried plant contained 0·27% alkaloids, principally jaconine, jacobine and seneciphylline. Changes in body weight, attributed to the accumulation of ascitic fluid, were seen after day 103. No significant gross lesions were observed in mice killed on days 63 and 129, but those dying or killed on days 133–193 showed ascites, involving fluid volumes of 2–25 ml, and slightly shrunken livers. All livers of mice examined after 129 or more days of feeding showed megalocytosis of the hepatocytes, cytoplasmic nuclear invaginations and cytosegrosomes. Lungs examined after 129 days showed enlarged cells in the bronchial epithelium, large alveolar cells, and large macrophages with cytoplasmic invaginations. Epithelial cells of the kidney tubules were enlarged in the proximal region and in the loops of Henle.

A relative resistance to the effects of Senecio alkaloids has been observed in rabbits fed 5% dried *S. jacobaea* for 90 days and thereafter 10% for up to 263 days (Pierson *et al. Res. Commun. chem. Path. Pharmac.* 1977, **16**, 561). Mean total consumption of Senecio was 112·5% of initial body weight. There was no apparent increase in weight, as described in mice and rats, and a slight reduction in body weight was attributed solely to the unpalatability of the feed. Kidney, lung, intestine and myocardium showed no significant abnormalities, but hepatocytes in all the lobules of the liver appeared to be swollen, causing slight structural distortion. The cytoplasm of the hepatocytes was vacuolated and granular, and the nuclei were irregular. No fibrosis or bile-duct proliferation was seen. Despite the apparent resistance of this species, a 150-mg/kg ip injection of pyrrolizidine alkaloids isolated from *S. jacobaea* killed two rabbits within 24 hr, suggesting that the low susceptibility of the rabbit to oral administration of these alkaloids is due to poor absorption from the gut.

The effect of the alkaloids of *S. jacobaea* on liver microsomal mixed-function oxidases in the rat has been investigated by Shull *et al.* (*J. anim. Sci.* 1976, **43**, 1024). The extracted alkaloids were injected ip in a dose of 65 mg/kg, and the animals were killed in batches of five and their livers were prepared for microsomal-activity measurements after 1 and 24 hr and 6 and 57 days. The rate of production of pyrrole metabolites in these preparations reached a peak within 1 hr and then declined and after 24 hr the activities of aminopyrine *N*-demethylase and cytochrome *P*-450 were reduced. Microsomal protein was also reduced at 24 hr. These results suggested that metabolically produced pyrroles reacted directly with enzymes to inhibit their own production and had a more delayed effect on *N*-demethylase activity and cytochrome *P*-450 either by depressing protein synthesis or by increasing protein degradation. In this way, prior exposure to plants containing pyrrolizidine alkaloids may reduce the damage to livestock subsequently exposed to them again.

The same investigators (Buckmaster *et al. ibid* 1976, **43**, 464) have shown that dietary cysteine gives rats some protection against the toxic effects of pyrrolizidine alkaloids. Dried *S. jacobaea* and *S. vulgaris* containing 0·181 and 0·206% total alkaloids, respectively, were fed at a dietary level of 5%. Survival time was shorter with *S. vulgaris* than with *S. jacobaea* and the acute ip toxicity of the alkaloids isolated from *S. vul-*

garis was greater than that of alkaloids from *S. jacobaea*. In the feeding tests, the addition of 1% cysteine to the diet improved the survival rate, whereas 1% methionine had no effect. Similarly, the percentage mortality in rats within 7 days of an ip injection of *S. jacobaea* alkaloids was reduced by the feeding of diet supplemented with 1% cysteine.

Factors affecting metabolism

Incubation of hepatic microsomal preparations from various animals with the alkaloid monocrotaline or with *S. jacobaea* alkaloids showed that pyrrole production diminished 19-fold, in the descending order male hamster, male rabbit, male mouse, male rat, beef steer, beef bull, female rat, wether lamb, male chicken and male Japanese quail (Shull *et al. ibid* 1976, **43**, 1247). In all the species except the rabbit and chicken, a direct relationship appeared between the rate of pyrrole production by the microsomal preparation and the *in vivo* susceptibility of that species to pyrrolizidine alkaloids. Since *S. jacobaea* was more toxic to rats after it has been incubated with sheep rumen fluid than after its incubation with cattle rumen fluid, the fact that sheep showed a greater resistance to the alkaloids than did cattle may reflect a difference in microsomal alkaloid metabolism rather than any detoxifying activity of the rumen. Rats given a single ip injection of 70 mg Senecio alkaloids/kg showed no change in total sulphydryl levels after 1 or 2 hr. Pretreatment of rats with 1% cysteine in the diet for 10 days also had no effect on liver sulphydryl levels, but when such rats were injected ip with 100 mg alkaloid/kg they had less bound pyrrole after 2 hr than similarly injected rats on a basal diet. These results, while supporting other indications of the protective effect of cysteine, demonstrate that there is no prolonged sulphydryl depletion as a result of the metabolism of pyrrolizidine alkaloids by the liver, thus undermining the view that the cysteine protection is due to its conjugation with pyrroles and the consequent reduction of their conjugation with essential cellular components, including enzyme sulphydryls.

Transfer of alkaloids in milk

The possibility that pyrrolizidine alkaloids might be transferred from cows to calves has been investigated by Dickinson *et al.* (*J. Am. vet. med. Ass.* 1976, **169**, 1192). Four cows were given dried *S. jacobaea* in doses of 10 g/kg/day by rumen cannula for 2 wk. Chromatography of the dried material showed the presence of jacobine, seneciphylline (jacodine), jacoline, jaconine and jacozine, accounting together, on average, for about 0·16% of the plant weight. The animals lost weight, their milk output declined and persistent diarrhoea developed after 7–14 days and continued until death. The calves gained weight normally. The only alkaloid detected in the milk from treated cows was jacoline, in a concentration of 9·4–16·7 μg/100 ml. In the cows, plasma albumin concentration declined and there was an unexplained leucocytosis. Blood sorbitol-dehydrogenase activity increased prior to the appearance of severe liver lesions (notably fibroplasia and megalocytosis). Liver biopsies revealed cellular changes but did not offer any particular advantage over the change in sorbitol-dehydrogenase activity as an index of liver dysfunction. Post-mortem findings in the cows were confined to liver lesions, and were typical of pyrrolizidine toxicosis. At present the metabolic fate of jacoline in calves drinking the affected milk is unknown.

The Senecio alkaloids

High-pressure liquid chromatography (HPLC) of a chloroform extract of an ammoniacal solution has been used to identify the active principles of *S. jacobaea* (Segall, *Toxicology Lett.* 1978, **1**, 279). Peaks representing only a single pyrrolizidine alkaloid were further analysed by mass spectrometry, while gas chromatography–mass spectrometry was used for peaks containing mixtures of alkaloids. These techniques demonstrated the presence of jacoline (mass/charge ratio 369), jaconine (*m/e* 387), seneciphylline (*m/e* 333), senecionine (*m/e* 335), jacozine (*m/e* 349) and possibly jacobine (*m/e* 351), the two latter being found in mixed HPLC peaks, together with isomers. An *m/e* 385 peak suggested the presence of an alkaloid closely related to jaconine. No pyrrolizidine alkaloid of *m/e* 305, such as has been reported elsewhere, could be detected. In *S. longilobus*, a plant that has been involved in cases of human poisoning arising from the use of herb teas in the USA, the alkaloids ridelline (*m/e* 349), retrorsine (*m/e* 351), seneciphylline (*m/e* 333) and senecionine (*m/e* 335) have been found (Segall & Molyneux, *Res. Commun. chem. Path. Pharmac.* 1978, **19**, 545).

A case of human poisoning attributed to *S. longilobus* led Huxtable *et al.* (*Proc. West. pharmac. Soc.* 1977, **20**, 455) to analyse an extract of the plant by thin-layer chromatography. This showed the presence of 0·3% pyrrolizidine alkaloids and 1·0% pyrrolizidine *N*-oxides, characterized as seneciphylline, senecionine and retrorsine *N*-oxide. The case in question, also described by Stillman *et al.* (*Gastroenterology* 1977, **73**, 349), concerned a girl aged 6 months who was given 8 oz of an infusion of 1·5 tablespoonfuls of *S. longilobus* in 16 oz boiling water on alternate days for 2 wk. She then started vomiting and was found to be ascitic, with a right plural effusion. Liver biopsy showed dilated sinusoids distended with mature erythrocytes; 2 months later there was extensive hepatocyte destruction, fibrosis and collagen deposition in the space of Disse.

A second, and fatal, case of *S. longilobus* poisoning was subsequently reported by the same group (Fox *et al. J. Pediat.* 1978, **93**, 980). This concerned a 2-month-old Mexican–American boy, who had been given herbal tea for 4 days to treat nasal congestion. The *S. longilobus* used for the infusion contained 1·5% (w/w) pyrrolizidine alkaloids, the probable total intake of which was calculated to be about 66 mg. The infant became progressively ill and lethargic; vomiting and haematemesis were followed after 24 hr by jaundice and indications of ascites, and the next three days saw the onset of seizures, a progressive loss of consciousness and of deep tendon reflexes, periods of apnoea, hypothermia, oliguria, high blood levels of sodium, potassium and calcium and persistent hyperbilirubinaemia. Cardiac arrest and death occurred on day 6. Although in the early stages the child's condition suggested Reye's syndrome, subsequent developments were not typical of this disease, and the autopsy findings, which included severe centrilobular

necrosis and fat accumulation in the liver, pulmonary oedema, atelectasis and necrotizing vasculitis, were similar to those associated with pyrrolizidine alkaloid poisoning in animals. The circulating free bilirubin and kernicterus in this infant were not typical of such poisoning and their cause was not established, but the kernicterus may well have been responsible for the encephalopathy.

Since bees may gather nectar and pollen from flowers of *S. jacobaea* suspected ragwort honey was examined for pyrrolizidine alkaloids by Deinzer *et al.* (*Science, N.Y.* 1977, **195**, 497). Senecionine, seneciphylline, jacobine, jaconine, jacoline and jacozine were all detected, together with one unidentified alkaloid. Concentrations of total pyrrolizidine alkaloids in a number of honey samples shown to contain some ragwort pollen were found to range from 0·3 to 4 ppm. It is unlikely, however, that any honey consumer would take enough to provoke toxic effects, particularly since ragwort honey is bitter and of poor colour and consequently is seldom marketed. Nevertheless, the long-term consumption of foods contaminated with these alkaloids is a potential problem. Livestock poisoning following consumption of ragwort and related plants is well known and the possible transfer of these alkaloids or their metabolites to consumers of meat and dairy products cannot be discounted.

[P. Cooper—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

Relatives of cyclamate

Spillane, W. J. & Benson, G. A. (1978). Metabolic studies of the non-nutritive sweeteners cyclopentylmethylsulfamate and cyclopentylsulfamate: determination of metabolites in rat urine. *J. pharm. Sci.* **67**, 226.

Cyclopentylmethylsulphamate (CPMS), cyclopentylsulphamate (CPS) and cycloheptylsulphamate (CHS) are non-nutritive sweeteners chemically related to cyclamate. The authors cited above have previously described studies in which single oral doses of CPS and CHS were administered to rats and rabbits. Some breakdown of the two sulphamates to their amine, alcohol and ketone metabolites occurred in both species (Benson & Spillane, *J. pharm. Sci.* 1976, **65**, 1841; *idem, ibid* 1977, **66**, 881). In the study cited above, similar investigations were carried out into the effects of administering a single dose of CPMS to rats and of extended feeding of CPS.

Sodium CPMS in aqueous solution was administered to five female Wistar rats in a single oral dose of of 1450 mg/kg. Urine was collected for 3 days after dosing. The urine samples were analysed for cyclopentylmethylamine and cyclopentylmethanol by gas–liquid chromatography (GLC). A spectrophotometric method was used to analyse for sulphamates. Neither of the sulphamates was converted to its amine, alcohol or ketone derivatives during storage in solution in urine for 3 days under refrigeration.

Three of the rats fed CPMS excreted cyclopentylmethylamine and cyclopentylmethanol. The mean conversions of the sulphamate to its amine and alcohol metabolites were 0·011 and 0·012%. In previous studies the mean conversions of CPS and CHS to cyclopentylamine and cycloheptylamine were 0·57 and 0·064%, respectively (Benson & Spillane, 1976 & 1977 *loc. cit.*). Therefore CPMS appears more stable than either of these other sulphamates. However, levels of cyclamate (cyclohexylsulphamate) conversion to cyclohexylamine in rats not pretreated with cyclamate are generally reported to be lower (Renwick & Williams, *Biochem. J.* 1972, **129**, 869; Suenaga *et al. Chem. pharm. Bull., Tokyo* 1972, **20**, 1357). The mean level of excretion of unchanged CPMS in the urine was 15·4%. For CPS and CHS, the amounts of a single dose recovered unchanged in the urine were reported to be 15 and 35%, respectively (Benson & Spillane, 1976 & 1977 *loc. cit.*).

Some metabolism of CPS occurred in all rats fed the compound over 9 days. Following administration of 200 mg on days 1–5 and on days 8 and 9 to five females, cyclopentylamine was excreted in the urine of all rats on day 1, but on the following 6 days there were considerable differences between individual animals in the levels of cyclopentylamine, cyclopentanol and cyclopentanone excreted. When feeding was resumed on days 8 and 9 only traces of metabolites were detected in the urine of all the rats.

ANTIOXIDANTS

Another try at BHT metabolism in man

Wiebe, L. I., Mercer, J. R. & Ryan, A. J. (1978). Urinary metabolites of 3,5-di-(1-[^{13}C]methyl-1-methylethyl)-4-hydroxytoluene (BHT-^{13}C) in man. *Drug Metab. Dispos.* **6**, 296.

It has been reported that the major urinary metabolite of BHT in man is 4-carboxy-2-(1-carboxy-1-methylethyl)-6-(1-formyl-1-methylethyl)phenol (*Cited in F.C.T.* 1968, **6**, 533). However, other workers have been unable to detect this compound, but have reported the presence of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT-acid) in human urine (*ibid* 1971, **9**, 296; Ryan, *Fd Cosmet. Toxicol.* 1971, **9**, 769). Further work on BHT metabolism is described below.

On three occasions, separated by intervals of 2 months, a single volunteer was given an oral dose of BHT suspended in olive oil. In one experiment 201·7 mg [^{13}C]BHT was taken, and in the other two, unlabelled material was administered at doses of 100 and 200 mg respectively. Urine was collected for 72 hr after dosing.

The major urinary metabolite was shown to have a mass spectrum comparable to the dicarboxylic acid aldehyde reported earlier (*Cited in F.C.T.* 1968, **6**, 533). However analysis by proton and ^{13}C-nuclear magnetic resonance spectroscopy indicated that the metabolite was in fact a dicarboxylic acid hemiacetal, 5-carboxy-7-(1-carboxy-1-methylethyl)-3,3-dimethyl-2-hydroxy-2,3-dihydrobenzofuran. The acetylated dimethyl derivative of the hemiacetal proved amenable to quantification by gas chromatography (GC). Over a period of 72 hr, 21·4% of the radioactive dose of [^{13}C]BHT was excreted in the urine as the hemiacetal. This metabolite was present in the urine samples collected during the first 3 hr after dosing, and excretion was at a maximum in the period 3–6 hr after BHT administration. Although the GC detection limit for BHT and its metabolites was of the order of 1 ng/ml of urine or whole blood extracted, the only other urinary metabolite identified was a small

amount of the BHT-acid (0·3% of the administered dose over 72 hr), and traces of *S*-(3,5-di-1-methyl-1-methylethyl-4-hydroxybenzyl)-*N*-acetylcysteine (mercapturic acid) were indicated in one fraction. Trace quantities of unchanged BHT, but no free metabolites, were found in serum samples taken at 15-min intervals up to 90 min after administration of 100 mg BHT. The total blood content of unchanged BHT (estimated on 4 litres of whole blood) ranged from 0·3 to 3·0 μg.

The low recovery of the adminstered dose of radioactivity indicated that urinary elimination is not the only route of elimination for BHT in man—a finding at variance with an earlier tracer study (Daniel *et al. Fd Cosmet. Toxicol.* 1967, **5**, 475)—while the rapid disappearance of BHT from the blood suggested that hepatobiliary excretion may be an important factor.

[This preliminary study may provide the much needed spur for further investigation of BHT's complex pharmacokinetics in both man and animals. The need for further studies was underlined by a FASEB Report to the FDA (*Fd chem. News* 1977, 19 (11), 17) which called for metabolic studies on the antioxidant to find a suitable species for evaluating the possible hazards of BHT to man.]

MISCELLANEOUS DIRECT ADDITIVES

A talc-on-rice survey

Stemmermann, G. N. & Kolonel, L. N. (1978). Talc-coated rice as a risk factor for stomach cancer. *Am. J. clin. Nutr.* **31**, 2017.

The coating of rice with talc is an accepted way to slow down spoilage and prolong shelf life, and consumers seem to prefer to buy rice that has a sheen (the rice is actually coated with glucose syrup and talc). In Hawaii, 85–90% of the rice sold is talc-coated. On the other hand, the sale of coated rice has been prohibited in Japan since 1947. These two areas have thus provided some opportunity for investigation of the hypothesis that the high incidence of stomach cancer among Japanese may be associated with the consumption of talc-coated rice, or more specifically of rice coated with asbestos-contaminated talc. Already-reported studies of the considerable Japanese population living in Hawaii (Haenszel *et al. J. natn. Cancer Inst.* 1972, **49**, 969) and of indigenous Japanese, in Miyagi and Okayama (Waterhouse *et al. Cancer Incidence in Five Continents*, Vol. III, p. 498; IARC, Lyon, 1976), like more recent studies carried out elsewhere (Cuello *et al. J. natn. Cancer Inst.* 1976, **57**, 1015), provided little support for this hypothesis.

Further light was thrown on the question when the Epidemiology Unit at the University of Hawaii conducted a nutritional survey among the various races in Hawaii on the basis of an annual random sample of 3% of the Hawaiian state population (Stemmermann & Kolonel, cited above). Questioning about weekly rice consumption revealed that the highest consumers of rice were the Filipinos, who had the lowest incidence of gastric cancer, and the next were the Japanese, who showed the highest incidence of this type of tumour. The survey thus emphasized the earlier lack of support for the suggestion that talc-coated rice might be an important factor in the aetiology of gastric cancer.

It is interesting to note that the high rate of gastric cancer among indigenous Japanese seemed to persist at a fairly stable level between the early 1960s and 1970s, during which period there was some decrease in the occurrence of this tumour among the Hawaiian Japanese.

AGRICULTURAL CHEMICALS

The renal effects of diphenylamine

Evan, A. P., Hong, S. K., Gardner, K., Jr., Park, Y. S. & Itagaki, R. (1978). Evolution of the collecting tubular lesion in diphenylamine-induced renal disease. *Lab. Invest.* **38**, 244.

Cystic dilation of the renal tubules, invariably accompanied by chronic interstitial nephritis, was found in rats fed diphenylamine (DPA) at dietary levels of 0·1% or more (*Cited in F.C.T.* 1968, **6**, 289). A 2-yr-old sample of DPA had far more severe renal effects than a freshly-manufactured sample, and a contaminant of the former was identified as the causative agent (*ibid* 1973, **11**, 334). The DPA-induced development of functional and structural changes in the rat kidney has now been followed in detail.

When rats were fed 1% dietary DPA for up to 78 wk, a decrease in urinary osmolality was evident after 2 wk, and was statistically significant at wk 6 and 20. The decrease in urinary osmolality was primarily caused by a decrease in the urinary concentration of urea and was accompanied by an increase in urine flow. Glomerular filtration, measured by endogenous creatinine clearance, was similar to that of the controls. After 5 wk, the medullary collecting tubules showed hyperplasia, with multilayering of cells, an increase in dark cells, and an increased number of [^{3}H]thymidine-labelled nuclei. By wk 10 some collecting ducts were dilated, with focal areas of cellular necrosis, and a few contained small amounts of cast material. These changes were far more frequent after 15–20 wk, and by wk 24 there were frank cysts in the cortex and medulla. After 52–78 wk such cysts were found in almost every segment of the nephron and the collecting tubules con-

tained many large cysts, which were usually filled with necrotic cast material. There were numerous areas of chronic inflammation with a concomitant loss of nephrons.

The findings suggested that hyperplasia and blockage of the connecting tubules with necrotic debris caused progressive dilation proximal to the obstructions, leading in time to cyst formation. The later stages of the condition observed in rats in this study resembled renal polycystic disease in man, but whether the initial stages of the disease are also similar is not yet known.

THE CHEMICAL ENVIRONMENT

Getting rid of arsenic

Charbonneau, S. M., Spencer, K., Bryce, F. & Sandi, E. (1978). Arsenic excretion by monkeys dosed with arsenic-containing fish or with organic arsenic. *Bull. env. contam. & Toxicol.* (*U.S.*) **20,** 470.

Arsenic (As) in the environment is particularly significant, since it is recognized that the regular intake of inorganic As may be followed by the development of internal malignant tumours as well as by keratoses (*Cited in F.C.T.* 1979, **17,** 309). Retention may be a factor determining As induction of tumours (*ibid* 1976, **14,** 507). Since some edible marine fish and shellfish contain relatively high concentrations of As, it is important to determine whether retention of As from such sources differs from retention of inorganic As.

An As balance study was undertaken in monkeys to determine the excretion pattern of As administered in fish. Four female adult cynomolgus monkeys were fed a single test meal of Atlantic grey sole (*Glypocephalus cynoglossus*) containing 77 ppm As, equivalent to about 1 mg As/kg body weight. The urine and faeces were collected every 24 hr for 14 days and the total As content of each sample was determined. After a 2-wk interval the experiment was repeated using As_2O_3 in an equivalent dose.

After administration of As in fish, 67% of the dose appeared in the urine within 14 days, almost all of it during the first 4 days. Except in one monkey, a small but significant amount was recovered from the faeces on days 2 and 3 (the total after 14 days being about 10% of the dose). Inorganic As was more rapidly excreted in the urine, about 60% of the dose appearing within 24 hr and 73% within 14 days in three of the four animals. Faecal excretion of inorganic As was less than 4% after 14 days, and urinary excretion of As had returned to background levels by day 11.

[Although these results indicate that more of the fish As is eliminated in the faeces, the significance of this result is doubtful because of the large inter-animal variations.]

Male view of acrylamide

Shiraishi, Y. (1978). Chromosome aberrations induced by monomeric acrylamide in bone marrow and germ cells of mice. *Mutation Res.* **57,** 313.

The effects of acrylamide on the nervous system are well documented (Thompson, *Fd Cosmet. Toxicol.* 1978, **16,** 188) but there is relatively little information on the teratogenicity or *in vivo* mutagenicity of this compound. In a study on pregnant rats (*Cited in F.C.T.* 1977, **15,** 154), the developing foetus was shown to be unaffected even at doses that produced neuropathy in the mother. However, a material can also exert an effect on reproduction via the male of the species; the present study in which acrylamide demonstrated a marked cytogenetic effect on the germinal cells of male mice (but a much lower activity against somatic cells) indicates that further investigations of this possible effect of acrylamide are warranted.

Acrylamide was administered to male mice either in the diet at 500 ppm for 1, 2 or 3 wk or by a single ip injection of 50 or 100 mg/kg. (Higher ip doses were tested but resulted in a high death rate.) In the injection studies, the bone marrow, spermatogonia and spermatocytes were examined 11 and 12 days after treatment, with the first two cell types also being sampled after 12 and 24 hr. All three cell types were examined immediately after the period of oral administration.

Acrylamide treatment was not associated with any changes in the numbers of sister-chromatid exchanges or chromosome aberrations such as dicentrics or rings in the bone-marrow cells. In both the oral and the parental studies, however, there was a time-related increase in the number of aneuploid and polyploid cells in the bone marrow; a more pronounced effect was seen in the spermatogonia. In addition, the number of spermatogonia with chromosome breaks increased from 2·4% in the controls to 19% after treatment for 21 days, and there was a small increase in cells with clear chromatid exchanges (0% in controls; 6% after treatment for 21 days). Acrylamide demonstrated a similar activity when given by injection. Examination 11 or 12 days after an ip dose of 100 mg/kg revealed an incidence of chromosome breaks in the spermatogonia three to four times greater than that in the controls. At this dose the mitotic index of the spermatogonia was reduced to such an extent that examination of the chromosomes after 12 and 24 hr proved impossible. Nevertheless, no aberrations were seen in the small sample of mitotic cells taken from mice treated 12 or 24 hr before with an ip injection of 50 mg/kg. No sister-chromatid exchanges were observed after injection of acrylamide at either dose level.

The spermatocytes may also be susceptible to the cytogenetic activity of acrylamide. A significantly higher level of aberrant chromosomes, particularly chain and ring quadrivalents and fragments, was observed after 2 and 3 wk of oral treatment and 11 or 12 days after a single ip injection of 100 mg/kg.

Testis weights were significantly decreased after 3 wk of oral administration and 11 or 12 days after injection of 100 mg acrylamide/kg.

Since the inhibition of mitosis masked early cytogenetic damage, it was uncertain whether the observed aberrations in the spermatocytes were a result of a direct toxic action or a consequence of the acrylamide-related changes in the spermatogonia. Acrylamide has been shown to alkylate the sulphydryl groups of proteins (Cavins & Friedman, *Proc. Fedn Am. Socs exp. Biol.* 1967, **26,** 822) and, in general, alkylating agents induce in spermatogonia chromatid aberrations that are not transmitted to the spermatocytes. Of greater concern to man is the possible transmission of the spermatocyte aberrations; the authors consider that further investigations should be undertaken in this area.

The subacute effects of acrylonitrile

Sukurai, H., Onodera, M., Utsunomiya, T., Minakuchi, H., Iwai, H. & Matsumura, H. (1978). Health effects of acrylonitrile in acrylic fibre factories. *Br. J. ind. Med.* **35,** 219.

On the basis of evidence that acrylonitrile (AN) is carcinogenic in rats, and possibly also in man, the US Occupational Safety and Health Administration has recently reduced the permissible time-weighted average exposure level in industry from 20 to 2 ppm (*Federal Register* 1978, **43,** 2586 & 45762). A similar reduction, to be implemented by 1981, has just been announced by the Health and Safety Executive in the UK, a level of 5 ppm being set for the intervening period. Earlier reports of adverse effects in workers had suggested that local irritation and mild liver damage might result from subacute exposure, but the levels involved were not precisely defined. The present investigation was undertaken, therefore, in an attempt to identify the exposure levels that might cause these subacute effects.

Medical examinations were performed on 102 workers who had been exposed to AN for more than 5 yr in six Japanese acrylic-fibre factories. These factories were divided into three groups on the basis of AN exposure levels; spot workplace samples indicated average levels in these groups of 2·1, 7·4 and 14·1 ppm AN, respectively. For AN in air collected by personal samplers, however, the group averages were far lower, at 0·1, 0·5 and 4·2 ppm, respectively. For the workers in these three factory groups, average urinary concentrations were, respectively, 3·9, 19·7 and 359·6 μg AN/litre and 4·5, 5·8 and 11·4 mg thiocyanate/litre. These 102 workers were compared with 62 controls employed on production lines not involving AN exposure. The urines of these controls were free of acrylonitrile and had a mean thiocyanate level of 4·0 mg/litre.

Many of the AN-exposed subjects had experienced irritation of the conjunctiva and upper respiratory tract, and some had developed scrotal irritation while working inside polymerization tanks and wearing respirators, but such complaints were infrequent by the time of the survey. Although the AN-exposed groups still showed a somewhat higher incidence of reddening of the conjunctiva or pharynx and of rashes or skin pigmentation, the differences from the control were not statistically significant. Extensive clinical chemistry investigations, in which special attention was paid to possible indications of disturbed liver function, revealed no consistent or dose-related differences of significance between AN-exposed workers and the controls. A palpable liver was considerably more frequent in all three AN-exposed groups, but no supportive evidence of impaired liver function was found in 20 workers so affected. No AN-related effects were apparent in other clinical tests, such as the measurement of blood pressure and neurological investigations.

Although the study failed to reveal any clear evidence of AN toxicity, it was thought possible that the increases in irritation and the occurrence of palpable liver might have been statistically significant in the most severely exposed group if a larger sample had been examined. An earlier survey carried out in the same factories had indicated that an increase in subjective complaints and in abnormal liver function was directly related to time spent in jobs involving AN exposure (Sakurai & Kusumoto, *J. Sci. Labour* 1972, **48,** 273), but working conditions had since improved considerably.

More on acrylonitrile

Thiess, A. M. & Fleig, I. (1978). Analysis of chromosomes of workers exposed to acrylonitrile. *Arch. Tox.* **41,** 149.

de Meester, C., Poncelet, F., Roberfroid, M. & Mercier, M. (1978). Mutagenic activity of acrylonitrile. A preliminary study. *Archs int. Physiol. Biochim.* **86,** 418.

A study of workers exposed to acrylonitrile (ACN) has suggested a possible carcinogenic hazard in industrial situations (*Fd chem. News* 1977, **19** (11), 26). In an Ames test, AN was mutagenic after metabolic activation, positive results being obtained at monomer levels as low as 57 ppm in the air (*Cited in F.C.T.* 1979, **17,** 179). Mutagenicity was also demonstrated in some strains of *Escherichia coli* (*ibid* 1979, **17,** 179).

Thiess and Fleig (cited above) prepared lymphocyte cultures from 18 workers who had been exposed to AN for an average of 15·3 yr and from an age-matched control group of 18 who had been exposed neither to AN nor to any substance suspected of damaging chromosomes. The exposed group was drawn from plant units manufacturing styrene–acrylonitrile co-polymers. Concentrations of AN recorded in the working atmosphere were generally about 5 ppm and were spasmodically higher during the decade prior to 1975, when these levels were reduced to 1·5 ppm.

For each person, 100 metaphases were analysed and photographically recorded. The incidence of aberrant metaphases varied from 0 to 4% (excluding gaps) or from 2 to 12% (including gaps) in the exposed group, while the corresponding ranges in the control group were 0–2 and 2–9%. The mean incidences of aberrant metaphases were 5·5 and 5·1% (including gaps) and 1·8 and 2·0% (excluding gaps) in the exposed and control workers, respectively. These figures provide no evidence of AN-induced damage to lymphocyte chromosomes in these workers.

The results of a mortality and morbidity study underway at the plants are awaited with interest.

Further evidence that the mutagenicity of AN in *Salmonella typhimurium* is microsome-mediated has been presented by de Meester *et al.* (cited above). Exposure of the Ames strains TA98, TA100, TA1530, TA1535, TA1537 and TA1538, with and without the S-9 fraction of rat-liver homogenate, to AN vapour under a variety of experimental conditions demonstrated that in the absence of metabolic activity, AN had no mutagenic effect upon any of the tested strains. In the presence of S-9 (derived from control rats and from rats treated with Aroclor 1254 or phenobarbital) at a concentration of 300 μl/ml, the number of revertant colonies increased significantly with strains TA1530 and TA1535 subjected to a concentration in the agar of 200 μg AN/plate, following exposure to 0·2% AN vapour for 1 hr. The reversion rate varied with the S-9 preparation. The mutagenic effect was weaker with strains TA100, TA1538 and TA98 and no effect was found with TA1537.

A bacterial fluctuation test detected mutagenic activity in TA1530 in the presence of the microsomal systems at concentrations ranging from 2·5 to 25 μg/ml.

Benzylamine metabolism

Wood, S. G., Al-Ani, M. R. & Lawson, A. (1978). Hippuric acid excretion after benzylamine ingestion in man. *Br. J. ind. Med.* **35**, 230.

A century ago the conversion of benzylamine to hippuric acid was demonstrated in the dog (Schmiedeberg, *Arch. exp. Path. Pharmak.* 1877, **8**, 1). In spite of the widespread use of benzylamine in industry, little is known of its biological fate in man, although Richter (*Biochem. J.* 1938, **32**, 1763) detected no excretion of unchanged benzylamine in human studies involving administration of oral doses of 160 mg. The authors of the above-cited study decided, therefore, to look at the metabolism using [^{14}C]benzylamine hydrochloride.

[^{14}C]Benzylamine hydrochloride (20 μCi) was administered orally to two fasting male volunteers. Samples of urine collected for the first 24 hr were either prepared directly for liquid scintillation counting or subjected to thin-layer chromatography (TLC). Areas corresponding to benzylamine, hippuric acid and benzoic acid were scraped from the TLC plates and counted.

The radioactivity detected in the urine by both methods was associated solely with hippuric acid. In 24 hr, over 98% of the radioactivity administered to both subjects had been recovered in the urine, 90% of the dose having been excreted within the first 3 hr. The authors concluded from the study that benzylamine was rapidly metabolized, probably via benzaldehyde, to benzoic acid and excreted as hippuric acid. The metabolism of benzylamine to benzoic acid was shown to be a rapid process, a point to be taken into account in any assessment of the overall toxicity of the compound, which is known to have potential as a mucosal irritant.

The non-carcinogenicity of diarylide yellow pigments

Leuschner, F. (1978). Carcinogenicity studies on different diarylide yellow pigments in mice and rats. *Toxicology Lett.* **2**, 253.

No evidence of carcinogenicity was found in an NCI bioassay of diarylanilide yellow, otherwise known as C.I. Pigment Yellow 12 (*Federal Register* 1978, **43**, 11760) and an Ames test also gave negative results (Milby & Kay, *J. Toxicol. envir. Hlth* 1978, **4**, 31). This pigment is based on 3,3′-dichlorobenzidine (DCB), which has been shown to be carcinogenic in animals, although not so far in man. When C.I. Pigment Yellow 13, also based on DCB, was given to rabbits in a single oral dose of 50 mg/kg, some 25 μg DCB was found in the urine (Akiyama, *Jikeikai med. J.* 1970, **17**, 1). The present investigation is concerned with the carcinogenicity and/or metabolism of three DCB-based pigments and one based on 3,3′-dimethylbenzidine (DMB; *o*-tolidine), which like DCB is a known animal carcinogen.

C.I. Pigment Yellows 12 and 83 (both DCB-based) and C.I. Pigment Yellow 16 (DMB-based), each containing not more than 2 ppm DCB or DMB, were fed for 104 wk to groups of 100 rats and mice at dietary levels of 0·1, 0·3 and 0·9%, providing intakes equivalent to average dose levels of 215–1960 mg/kg for mice and 68–630 mg/kg for rats. Pigment Yellow 83 to which 20 ppm DCB had been added was also fed at the same levels. No adverse effects on growth, feed intake, general condition, survival, gross or histological appearance of the tissues or tumour incidence were detected in any of the treated groups. No DCB was found in the urine of rats after 6 or 23 months of treatment with the highest level of Pigment Yellows 12 or 83, even when the latter contained 20 ppm DCB, and DMB was similarly absent from the urine of rats treated with Pigment Yellow 16. When Pigment Yellow 13 was given to five rabbits in a single oral dose of 50 mg/kg, no DCB could be detected in the urine after 48 or 72 hr at a level of sensitivity of 0·3 ppm, using the same analytical methods as Akiyama (*loc. cit.*). It was concluded that none of these pigments is likely to present a risk of carcinogenicity to man.

Further infertility from dibromochloropropane

Biava, C. G., Smuckler, E. A. & Whorton, D. (1978). The testicular morphology of individuals exposed to dibromochloropropane. *Expl mol. Path.* **29**, 448.

Recently, 1,2-dibromo-3-chloropropane (DBCP) has been heralded as a possible cause of sterility among industrially exposed workers, and DBCP studies in the rat have demonstrated the testis to be one of the target organs (*Cited in F.C.T.* 1978, **16**, 498). In the above-cited study, testicular biopsy specimens were taken from ten men with histories of direct industrial exposure to DBCP. These samples were examined histologically by light and electron microscopy, and the findings revealed three fairly distinct groups.

Three patients (group 1), two of whom had undergone only brief periods of exposure (0·25 and 1·75 yr), showed a normal or slightly decreased formation of sperm, and the germ cells and Sertoli cells showed no distinct abnormalities. The two patients in group 2 had long histories of DBCP exposure (at least 10 yr) and had no spermatogenic activity. Microscopy revealed that there were no germ cells in the seminiferous tubules and there was no evidence of cellular necrosis or residual bodies either in the lumina of the seminiferous tubules or within the cytoplasm of the Sertoli cells. The third group consisted of five patients subjected to DBCP exposure for periods between these two extremes. These patients had reduced sperm counts, and spermatogenic cells were seen only in a minority of the seminiferous tubules. In the three more severely affected men in this group, spermatogenic activity was limited to a few short segments of the tubules. The Sertoli cells of the group-3 men were well preserved and appeared similar to those in group 2.

Although the limited number of patients prevented any statistical treatment of the data, the severity of reduction in spermatogenic activity correlated with the duration of exposure to DBCP. The mechanism of DBCP's action on the testis is unknown. Was the suppression of spermatogenesis due to a failure of the normal process responsible for the removal of spermatogonia at the stem-cell level? The histological findings in this study provide some support for this possibility. On the other hand DBCP could be acting primarily on the stem cells, which because of their position are more readily accessible to chemicals circulating in the blood than are the Sertoli cells. The latter are major components of the blood–testis barrier, providing a protected compartment within which the germ cells complete their division and differentiation.

The authors consider that critical analysis of a laboratory model is essential for the further assessment of the effects of DBCP.

EDTA and the rat intestine

Rosenblatt, D. E. & Aronson, A. L. (1978). Calcium ethylenediaminetetraacetate (CaEDTA) toxicity: time- and dose–response studies on intestinal DNA synthesis in the rat. *Expl mol. Path.* **28**, 202.

Rosenblatt, D. E., Doyle, D. G. & Aronson, A. L. (1978). Calcium ethylenediaminetetraacetate (CaEDTA) toxicity: time- and dose–response studies on intestinal morphology in the rat. *Expl mol. Path.* **28**, 215.

Calcium disodium ethylenediaminetetraacetate ($CaNa_2EDTA$) is used in small amounts as a food additive and is poorly absorbed from the gut (Joint FAO/WHO Expert Committee on Food Additives, F.A.O. Nutr. Mtg Rep. Ser. No. 40A,B,C; WHO/Food Add./67.29, p. 44). Injections of the salt have been used to treat lead poisoning in man and domestic animals, but they have also been shown to produce ultrastructural changes in the kidneys and morphological changes in the intestines of rats (*Cited in F.C.T.* 1978, **16**, 81).

The first study cited above shows the relationship between continuous iv infusion of $CaNa_2EDTA$ and the inhibition of intestinal DNA synthesis. In dose–response studies, rats were given an iv infusion over 24 hr of 0·75, 1·5, 3 or 6 mmol $CaNa_2EDTA$/kg/24 hr, and in time–response studies they were given an infusion of 6 mmol $CaNa_2EDTA$/kg/24 hr for 6, 12, 18 or 24 hr. In each group an ip injection of [*Me*-^{14}C]-thymidine (0·25 mCi/kg body weight) was given 1 hr before the animals were killed and its incorporation into intestinal DNA was determined. Significant inhibition of DNA synthesis occurred after infusion of 3 mmol $CaNa_2EDTA$/kg/24 hr. After a 24-hr infusion of 6 mmol/kg/24 hr, $CaNa_2EDTA$ inhibited DNA synthesis to a degree similar to that recorded 2 hr after an iv injection of cytosine arabinoside, a potent inhibitor of DNA synthesis. An iv injection of 25 mg folate/kg had no effect on $CaNa_2EDTA$-induced inhibition of DNA synthesis, despite previous indications (Taylor & Jones, *Biochem. Pharmac.* 1978, **21**, 3313) that folate might protect against this effect of $CaNa_2EDTA$.

When zinc- or cobalt-containing EDTA chelates (6 mmol/kg/24 hr) were administered for 24 hr, no inhibition of DNA synthesis occurred. Recovery of the ability of the intestine to synthesize DNA in rats dosed with 6 mmol $CaNa_2$ EDTA/kg/24 hr was enhanced by Zn supplementation but not by Co or Mn supplementation (acetate salts of the metals given iv; 12 μmol/rat).

The second study cited describes changes in intestinal morphology when rats were dosed with $CaNa_2EDTA$ as in the dose- and time–response experiments described above. A dose of 6 mmol/kg/24 hr infused over 24 hr was needed for marked morphological changes to occur—the villi were shortened, with a shortened epithelium and narrowed brush borders. The crypt epithelium was more cuboidal and contained basophilic bodies. There were increases in the numbers of lymphoid cells, particularly in the lamina propia, and of large mononuclear cells (possibly macrophages), but the number of plasma cells was lower than in the controls. The mitotic index was decreased. After a 36-hr infusion at 6 mmol $CaNa_2EDTA$/kg/24 hr, the villi were reduced to low humps with little or no brush border and no mitotic figures. The epithelium was cuboidal and absent in some places; epithelial cells contained clear vacuoles possibly caused by swollen organelles such as mitochondria. The overall effect was a general reduction in the number of cells specifically differentiated into villus, goblet and Paneth cells and an increase in the number of cells normally associated with an inflammatory response. When infusion was stopped after 36 hr the intestine began to recover and after a 48-hr recovery period the mitotic index was greater than in the controls. The pattern of changes indicated the inhibition of DNA synthesis by the chelating agent, in conjunction with an inflammatory response.

NATURAL PRODUCTS

Don't blame the alcohol, it could be the aldehyde

Ristow, H. & Obe, G. (1978). Acetaldehyde induces cross-links in DNA and causes sister-chromatid exchanges in human cells. *Mutation Res.* **58**, 115.

Relationships have been shown between alcoholism and the incidence of certain types of cancer (Lowenfels, *Ann. N.Y. Acad. Sci.* 1975, **252**, 366), although their possible significance is clouded by the frequent association of alcoholism with factors such as smoking and malnutrition. A positive dose–response relationship between ethanol and dominant lethal mutation has been demonstrated in mice (*Cited in F.C.T.* 1975, **13**, 581), and chromosomal aberrations have been detected following the administration of ethanol to grasshoppers (*ibid* 1965, **3**, 516). The authors cited above are of the opinion that the mutagenicity of ethanol may not be due to the alcohol itself, but to its primary metabolite, acetaldehyde. In this paper they describe studies which indicate that acetaldehyde induces cross-links in isolated-DNA strands and sister-chromatid exchanges in human lymphocytes.

The basis of the method used to study cross-linking of isolated DNA strands was that thermally denatured DNA will not significantly reanneal, under appropriate conditions and within a limited time, unless cross-links between the strands are present. Calf-thymus DNA (25 μg/ml) in sodium chloride/sodium citrate solution was incubated with 1 M-acetaldehyde for 30 min at 37°C and then dialysed against sodium chloride/sodium citrate overnight. A control DNA sample was incubated without acetaldehyde and dialysed. In order to determine hyperchromicity, the DNA was gradually heated to 90°C and the absorbance at 260 nm was recorded. The control DNA and acetaldehyde-treated DNA showed similar hyperchromicity and characteristic thermal transition ("melting") mid-points (T_m).

After reaching 90°C, the DNA was rapidly cooled and then the absorbance was measured again as it was re-heated. The acetaldehyde-treated DNA melted in a manner corresponding to that during the first heating, and with a very similar T_m, except that the hyperchromicity was reduced by about 40%. On re-heating, the control DNA had no characteristic T_m and the hyperchromicity was very much reduced. The authors suggest that these results indicate that DNA treated with acetaldehyde partially re-anneals after thermal denaturation and that this is caused by the acetaldehyde-induced formation of cross-links between complementary DNA strands.

In a second experiment calf-thymus DNA or highly purified *Bacillus brevis* DNA were incubated with acetaldehyde, as previously described, for up to 120 min and then hyperchromicity and the extent of re-annealing were determined as before. It was found that both initial hyperchromicity and re-annealing after rapid cooling were dependent on the duration of acetaldehyde treatment.

Different concentrations of acetaldehyde (0·0005, 0·001 and 0·002%, v/v) were added to cultures of human lymphocytes 24 hr (and with 0·002% also 48 hr) before cell fixation. Bromodeoxyuridine (10^{-5} M) was added 48 hr before fixation and colcemide (0·08 μg/ml) 4·5 hr before fixation. The chromosome preparations were stained to show sister-chromatid exchanges (SCE). There was a dose-dependent effect of acetaldehyde on SCE frequency and with 0·002% (v/v) acetaldehyde the SCE frequency was greater after 48 hr than after 24 hr.

Fibre and cancer—further evidence?

Freeman, H. J., Spiller, G. A. & Kim, Y. S. (1978). A double-blind study on the effect of purified cellulose dietary fiber on 1,2-dimethylhydrazine-induced rat colonic neoplasia. *Cancer Res.* **38**, 2912.

The incidence of colon cancer is higher in the industrialized areas of the world than in areas of lower socio-economic status, and epidemiological studies have strongly implicated some environmental factor or factors (*Cited in F.C.T.* 1976, **14**, 209). Of possible factors, diet has been most intensively investigated and there has been much debate about the role of dietary fibre. Experimental studies have given rise to conflicting results, but this may be because different types of fibre or chemically ill-defined fibre have been used (*ibid* 1976, **14**, 210). In the study cited above, the effect of one specific dietary-fibre component, microcrystalline cellulose, on dimethylhydrazine-induced neoplasia in rats was investigated.

Male Wistar rats (6 wk old), previously fed standard laboratory chow pellets, were randomly assigned to two groups each of 30 rats. Rats in both groups were fed on chemically-defined diets of equal calorific value, but the feed of one group contained 4·5% (w/w) highly purified microcrystalline cellulose and that of the other contained no dietary fibre (determined by analysis for cellulose, hemicellulose, lignin, pectin and mucilages). After 2 wk, dosing of animals with carcinogen began. Twenty animals in each dietary group were given sc injections of 1,2-dimethylhydrazine dihydrochloride (25 mg/kg) once a week for 16 wk. Every 4 wk, beginning 4 wk after the final injection, three or four randomly selected rats from each dietary group were killed and autopsied. Any neoplasms were examined histologically.

There were no statistically significant differences between the carcinogen-treated and untreated (control) groups or between the groups fed fibre- and non-fibre-containing diet with respect to weight gain, food and water intake, urine output or faeces production, except that the latter was greater in the fibre-fed group than in the non-fibre group. No colonic neoplasms were detected in the control animals of either dietary group. Colonic neoplasms were detected in 70% of the carcinogen-treated rats on the fibre-free diet, but in only 30% of the treated rats fed on a cellulose-containing diet. The average number of colonic tumours per rat was also lower in the cellulose-fed rats. The beneficial effect of cellulose fibre on colonic-tumour incidence appeared to be time-dependent since the reduction in tumour incidence did not become statistically significant until the final 8 wk of the study.

There were differences in the distribution of neoplasms between the two groups. In rats fed on a fibre-free diet, 27% of the tumours occurred in the proximal portion of the colon but only 12·5% were located in this area in rats fed on a cellulose-containing diet. There were no significant differences in the size or histopathology of colonic tumours in rats from the two groups, and the incidence of tumours of the ear and small intestine was similar in both groups.

The authors consider that these results provide good evidence that the intake of dietary cellulose fibre protects rats against the development of colonic tumours. Further studies would have to be carried out to determine whether this beneficial effect occurs only with cellulose and whether the effect with cellulose is due to added bulk in the diet or to some specific property in the fibre. However, if these results could be extrapolated to man they would support the view that lack of dietary fibre increases the risk of cancer of the colon.

Do-it-yourself nitrosation

Tannenbaum, S. R., Fett, D., Young, V. R., Land, P. D. & Bruce, W. R. (1978). Nitrite and nitrate are formed by endogenous synthesis in the human intestine. *Science, N.Y.* **200,** 1487.

Nitrosamines in foodstuffs may play an important role in human carcinogenesis (Archer & Wishnok, *Fd Cosmet. Toxicol.* 1977, **15,** 233; *Cited in F.C.T.* 1978, **16,** 627) and there is evidence that nitrosamines can be produced by the gut bacteria even at neutral pH (*ibid* 1971, **9,** 739). In an attempt to minimize the chance of nitrosation both of amino compounds in the diet and of endogenous amino compounds, suggestions have been made to reduce the amount of nitrite and nitrate in water and food. It appears, however, that both nitrite and nitrate can be formed *de novo* in the human intestine, thus thwarting to some degree attempts to minimize their presence in the body.

Six healthy subjects participated in a nitrate balance study. They were given a protein-free diet for 10 days, and then a diet providing 0·8 g egg protein/kg body weight/day for 10 days. Urinary and dietary components were assayed for nitrate and nitrite by a modified Griess microprocedure. Despite the expectation that the subjects would rapidly reach equilibrium and excrete a steady-state concentration of nitrate, they showed wide fluctuations in urinary nitrate from day to day during consumption of either diet. Some significant source of nitrate other than the diet was therefore postulated.

In a second study, eight healthy subjects on free-choice diets (about 43% fat, 18% protein and 39% carbohydrate) submitted single faecal samples, and another six who had undergone total colectomy submitted samples of ileostomy effluent. There were great variations in these samples, but all contained nitrite, the levels in the ileostomy samples being consistently high, while five of the eight normal faecal samples contained nitrate also. No nitrate occurred in the ileostomy effluents. Faecal samples from two of these individuals were analysed on several separate occasions; notwithstanding various changes in diet, nitrite was always present, although the detection of nitrate was spasmodic.

Heterotrophic nitrification of ammonia or organic nitrogen compounds may therefore take place in the upper, aerobic, portion of the human intestine. As the material passes through the intestine the increasingly anaerobic conditions lead to the accumulation of nitrate as well as nitrite. A proportion of these compounds may be utilized by other faecal microorganisms, some may be absorbed through the intestinal wall, and some may react in the relatively acidic portion of the caecum and colon to form *N*-nitroso compounds.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

Photoallergy to 6-methylcoumarin

Kaidbey, K. H. & Kligman, A. M. (1978). Contact photoallergy to 6-methylcoumarin in proprietary sunscreens. *Archs Derm.* **114,** 1709.

6-Methylcoumarin (MC), a widely-used cosmetic fragrance, was recently found capable of inducing contact photoallergy in human volunteers (Kaidbey & Kligman, *Contact Dermatitis* 1978, **4,** 277). A severe case of contact photoallergy which developed after application of two popular sunscreens containing MC is now reported.

A 22-yr-old woman holidaying in Jamaica applied a mixture of two sunscreens, respectively containing the active ingredients amyl dimethyl *p*-aminobenzoate (ADAB) and homomenthyl salicylate (HMS) to her face, chest and thighs. Intense erythema and pruritis had developed on these areas after 24 hr, and after 48 hr there were severe oedema, vesiculation and oozing. The woman had no history of sun sensitivity, and was not taking any drugs. Analysis of the two sunscreens revealed 0·032 and 0·2% MC respectively. When 1% MC, 5% ADAB, 3% HMS and the two sunscreens were separately applied under cover to the woman's back, and irradiated after 6 hr with long wavelength UV light, MC and the two sunscreens produced oedema and vesiculation, but no reaction resulted from ADAB or HMS. Both unirradiated sites treated with MC and the sunscreens and left covered for 48 hr and untreated skin irradiated with UV light gave negative results. Three other subjects known to be contact photosensitive to MC developed indurated and intensely pruritic reactions after photopatch testing with the two sunscreens, whereas 14 normal subjects were unaffected.

[The FDA has recently asked manufacturers of suntan preparations to remove MC, on the grounds that it may cause photosensitization (*FDA Consumer* 1979, **12** (10), 24).]

Monkeys with dandruff problems

Gibson, W. B. & Calvin, G. (1978). Percutaneous absorption of zinc pyridinethione in monkeys. *Toxic. appl. Pharmac.* **43**, 425.

Absorption, distribution and excretion studies to examine the fate of the anti-dandruff agent, zinc pyridinethione (ZPT), when given iv, dermally or orally to rabbits have previously been reported (*Cited in F.C.T.* 1977, **15**, 496). The results indicated that the organic and inorganic portions of ZPT dissociate prior to distribution in and excretion from the body. Only a small percentage of either portion penetrates the skin and the zinc does so to a lesser extent than the organic portion. The present study consisted of two series of experiments to estimate the systemic exposure to ZPT that could be expected from exaggerated topical application to primates.

Intact abdominal skin of rhesus monkeys was exposed to lotions that contained 2% ^{35}S-labelled ZPT, with or without surfactant, for 3 hr, either once or on three successive days. A single 3-hr application of the lotion, with surfactant, was also made on the abraded abdominal skin of one animal. Material remaining on the skin was removed after each 3-hr exposure. Absorption of ZPT was determined by summing the ^{35}S found in the urine, faeces and carcass after 60–80 mg of [^{35}S]ZPT had been applied to a 10 cm^2 area of skin.

When ZPT was applied once, with or without surfactant, to intact skin, the absorption was 0·02–0·04%. The absorption increased to 0·3% when abraded skin was treated with ZPT and surfactant. When applied to intact skin repeatedly (three times) without surfactant, absorption was again low (0·02%); after repeated application with surfactant, absorption increased to 0·2%. Concentrations of ^{35}S in the blood were below the threshold of detection (0·02 μg ZPT/g blood) except following repeated applications with surfactant or after a single application to abraded skin. The ^{35}S that was absorbed was almost entirely excreted in the urine and only small fractions were found in the faeces or in the tissues at autopsy.

The percutaneous penetration of ZPT through the scalp of rhesus monkeys was investigated by applying a suspension (400 μg/10 cm^2) of [^{14}C]ZPT in an aqueous solution of triethanolamine alkyl sulphate and allowing it to dry. The amounts of ^{14}C absorbed and subsequently excreted were small, and were similar whether the ZPT was in contact with the scalp for 3 or 72 hr. The absorption of ZPT, calculated from the radioactivity that occurred in the urine and faeces was in the range 1–5% of the applied dose in all four treated animals. Blood concentrations of ^{14}C were generally below the detectable limit (1 ng/g calculated as [^{14}C]ZPT).

It was concluded that penetration of ZPT through intact primate skin is very slight. When the integrity of the stratum corneum is disrupted either by abrasion or by repeated exposure to concentrated surfactant solutions, ZPT absorption is increased approximately tenfold, but even then only low levels of ZPT are absorbed. If human skin is similar to that of monkeys, with respect to ZPT penetration, the amount of ZPT absorbed from shampoos containing particulate ZPT is likely to be extremely small and probably without physiological significance.

In this study only one or two animals were used in each group, and the authors noted some variation between animals with regard to flux rates of radioactivity through the skin. They also emphasized that the observations were based on measurements of radioactivity, not of ZPT, and that the chemical identity of the labelled compounds in biological samples was not determined. It is concluded that the very low levels of radioactivity determined in many samples may have been due to impurities, and therefore all values cited should be considered as upper limits for ZPT absorption.

Talc in the ovaries

Henderson, W. J., Hamilton, T. C. & Griffiths, K. (1979). Talc in normal and malignant ovarian tissue. *Lancet* **i**, 499.

Some years ago it was reported that talc had been found deep within a small number of tumours of the ovary, uterine cervix and endometrium, as well as in five of 12 "normal" ovaries (Henderson *et al. J. Obstet. Gynaec. Br. Commonw.* 1971, **78**, 266). The talc was identified with an electron-microscope microanalyser, with combined solid-state energy dispersive detector and crystal spectrometers. The authors were confident that the talc could not have entered the tissues during processing, but as surgeons' gloves at the time were still sometimes contaminated with talc (*Cited in F.C.T.* 1977, **15**, 87) they have now conducted a further study of ovarian tissue removed with the use of talc-free gloves.

The tissue was handled by fine forceps only, and was analysed by an oxygen-incineration procedure. Talc was identified in all three normal ovaries, in three cystic ovaries and in three adenocarcinomas examined, in amounts ranging from 6900 to 55,100, from 17,400 to 24,300 and from 6400 to 24,500 particles/g tissue (wet weight), respectively.

[The process by which talc reaches the ovaries remains obscure, since no radioactivity was found in the ovaries of rabbits after six daily intravaginal doses of ^{3}H-labelled talc (Phillips *et al. Fd Cosmet. Toxicol.* 1978, **16**, 161). Moreover, the lack of any correlation between the levels present and the state of the ovaries in the above survey (albeit very limited) does not suggest that talc plays a role in ovarian cancer.]

TRIS under fire again

Furukawa, M., Sirianni, S. R., Tan, J. C. & Huang, C. C. (1978). Sister chromatid exchanges and growth inhibition induced by the flame retardant tris(2,3-dibromopropyl) phosphate in Chinese hamster cells: *Brief Communication. J. natn. Cancer Inst.* **60**, 1179.

Recent studies have shown that the flame retardant tris(2,3-dibromopropyl) phosphate (TRIS) is capable of altering the size of the DNA molecule obtained from human cells (*Cited in F.C.T.* 1979, **17**, 98). These results have added to the toxicological evidence that has resulted in restrictions on TRIS in various countries. Further condemning evidence comes with the

present studies on the effects of the compound on cell growth, chromosomal aberrations and sister-chromatid exchanges (SCE).

The effect of TRIS on cell growth was determined by examining the ability of Chinese hamster V79 cells to form colonies after exposure either to different doses of TRIS (0–200 μg/ml) for 3 hr or to 20 or 40 μg TRIS/ml for various periods (0–42 hr). Results indicated that TRIS caused a reduction in cell growth that was dependent both on the dose and on the duration of exposure. TRIS produced highly significant dose-dependent increases in SCE in V79 cells either cultured *in vitro* (0–20 μg TRIS/ml; 3-hr exposure) or in diffusion chambers implanted into mice (given 0–500 μg TRIS/g body weight). In contrast, increases observed in chromosomal aberrations *in vitro* (0–50 μg TRIS/ml; 3-, 6-, 18- or 24-hr exposure) or in diffusion chambers in mice (0–1000 μg TRIS/g body weight) were not significant. Also, no significant increase in chromosomal aberrations was observed in bone-marrow cells from mice receiving doses of TRIS (up to 1000 μg/g body weight) for 24, 48 or 72 hr. Similarly, when human lymphoid cell lines Jeff and B35M were treated with TRIS in the same doses and for the same exposure times as V79 cells *in vitro*, there was no increase in chromosomal aberrations.

The reasons for these contrasting results are unclear. It is possible that the TRIS became partially inactivated *in vivo* and as a result was not potent enough to induce chromosomal aberrations but was still sufficiently potent to induce SCE. Alternatively, it has been suggested that the mechanisms leading to SCE are not necessarily associated with the mechanisms of chromosomal aberrations, although both presumably result from initial damage to chromosomal DNA.

PATHOLOGY

Taking asbestos to heart

Churg, A., Warnock, M. L. & Bensch, K. G. (1978). Malignant mesothelioma arising after direct application of asbestos and fiber glass to the pericardium. *Am. Rev. resp. Dis.* **118**, 419.

With the widespread use of asbestos in industry, transport, building and home appliances, the asbestos fibre has become an important environmental contaminant and has been found in the lungs of people not occupationally exposed to it. The association between exposure to asbestos and the subsequent development of pleural and peritoneal mesotheliomas is well established and the above-cited study reports on a patient who, 16 yr before his death at the age of 62 yr, had a mixture of fibrous dusts, mainly asbestos with some fibre glass, dusted over his pericardial cavity.

The man (who had never smoked) developed angina pectoris in 1954 and 3 yr later underwent pericardiopexy to improve the collateral coronary circulation; the epicardial surface was dusted with 0·3 g asbestos (probably amphiboles) and fibre glass. He recovered and, free from symptoms, returned to work as an engineer in a paint factory, where he was exposed to lead dust.

Blood lead concentrations were within normal limits, but in 1971 a chest film indicated a large right pleural effusion, which on analysis showed many malignant cells. A later chest film showed the heart to be enlarged and, at thoracotomy, multiple small tumour nodules were seen on the visceral and parietal pleura and diaphragm. The biopsy finding was interpreted as a malignant mesothelioma, and in 1972 a tumour was treated with radiotherapy. In 1973, after severe chest pain and shortness of breath, the patient died.

An autopsy showed many subcutaneous masses over the chest wall, an extensive tumour mass binding the heart, lungs and diaphragm, and transparent fibres and ferruginous bodies within the pericardium. Examination by electron microscopy showed two thirds of the fibres to be tremolite and anthophyllite asbestos; the remainder were fibre glass.

The authors report this case as the first example of a human developing mesothelioma following direct contact between a mesothelial surface and fibrous dust.

CANCER RESEARCH

Agglutination test for bladder carcinogens

Kakizoe, T., Kawachi, T. & Okada, M. (1978). Concanavalin A agglutination of bladder cells of rats treated with bladder carcinogens; a rapid new test to detect bladder carcinogens. *Cancer Lett.* **5**, 285.

Several rapid screening tests for carcinogens have been developed (Grasso, *Fd Cosmet. Toxicol.* 1977, **15**, 74) but they do not detect organ-specific carcinogenesis. A rapid screening method for the detection of bladder carcinogens has now been reported.

N-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) and many related compounds have carcinogenic effects in rats, often specifically on the bladder, and a good correlation has been shown between their carcinogenicity to rats and their mutagenicity (Nagao *et al.* *Cancer Res.* 1977, **37**, 399). Plant lectin, concanavalin A (Con A), agglutinates transformed cells more effectively than normal cells *in vitro*, and this is the basis of the method described in the paper cited above.

Groups of five male Wistar rats were administered BBN or one of the following related compounds at equimolar concentrations in their drinking-water: *N*-butyl-*N*-(3-carboxylpropyl)nitrosamine (BCPN), *N*-ethyl-*N*-(4-hydroxybutyl)nitrosamine (EHBN), *N*-butyl-*N*-(3-hydroxyethyl)nitrosamine (BHEN), *N*-butyl-*N*-(3-hydroxypropyl)nitrosamine (BHPN) and *N*-*tert*-butyl-*N*-(4-hydroxybutyl)nitrosamine (*t*-BBN). The total doses administered per animal in each group were 110 mg BBN, 115 mg BCPN, 112 mg EHBN, 115 mg BHEN, 101 mg BHPN and 124 mg *t*-BBN. A control group of animals was given untreated deionized water. At the end of the week, the animals were killed and the bladders were removed, everted and washed in phosphate buffered saline. The bladder necks were ligated and mucosal cells were isolated by treatment with ethylenediaminetetraacetic acid and sonication. Cells from all the rats in a given treatment group were combined for agglutination assays carried out with Con A.

There were significant increases in agglutination by Con A with cells from rats treated with BBN, BCPN, EHBN and BHEN, compared with the agglutination of cells from control animals and animals treated with *t*-BBN and BHPN. BBN, BCPN and EHBN are known bladder carcinogens in rats, but *t*-BBN and BHPN are not. BHEN induces carcinogenesis of the liver and oesophagus in rats but has not yet been shown to produce bladder tumours, although it has been linked with the development of hyperplasia in the bladder mucosa.

The numbers of large superficial cells from the mucosal lining were lower in treated groups than in the controls, probably because of their loss by exfoliation during treatment. The authors claim that cell agglutination by Con A seemed to be closely related to the appearance of microvilli on the luminal surface. They report that preliminary experiments demonstrate Con A-agglutination of isolated mucosal cells from BBN-induced bladder cancers in rats and from human transitional-cell bladder tumours obtained by surgery. They consider that this technique can be used both for screening for potential bladder carcinogens and for the early diagnosis of human bladder cancer by the detection of preneoplastic changes.

[It is perhaps worth commenting again that results of tests for bladder carcinogens in rodents often do not correspond with those in other experimental animals, notably dogs, and are not necessarily indicative of chemical carcinogenesis in man (*Cited in F.C.T.* 1972, **10**, 121).]

Mechanisms of co-carcinogenesis

Lakowicz, J. R. & Hylden, J. L. (1978). Asbestos-mediated membrane uptake of benzo[*a*]pyrene observed by fluorescence spectroscopy. *Nature, Lond.* **275**, 446.

Ferric oxide (Fe_2O_3) has been implicated as a co-carcinogen in respiratory-tumour induction by benzo-[*a*]pyrene (BP), and it has been suggested that the co-carcinogenic effect may result from increased retention of BP in the respiratory tract (*Cited in F.C.T.* 1977, **15**, 499). Other studies have shown co-carcinogenic activity in the combined effects of cigarette smoking and the inhalation of asbestos fibres (*ibid* 1970, **8**, 209). In order to examine one possible mechanism of co-carcinogenesis, the ability of asbestos and silica to increase the rate of cellular uptake of BP has been investigated using model membrane systems and fluorescence spectroscopy.

Particulate samples were prepared by mixing the particulate with a benzene solution of BP and evaporating the benzene under pressure. Fluorescence emission spectra indicated that asbestos (in the amosite form) adsorbed more BP in the monomeric state than did silica, even though the surface area of the amosite was about 60 times less than that of the silica. (Surface areas were determined by nitrogen adsorption.)

Single bilayer vesicles of dipalmityl-L-α-phosphatidylcholine (DPPC), prepared by ultrasonic irradiation, were used as a model membrane. Either an amount of particulate containing 5 μg BP, or 5 μg microcrystalline BP, was suspended in 10 ml buffer, and 10 mg DPPC in 1 ml buffer was added. Uptake of BP into the phospholipid vesicles was more rapid from amosite-adsorbed BP than from silica-adsorbed BP. The rate of uptake of microcrystalline BP was slowest. When asbestos or silica was added to BP microcrystals suspended in buffer, the rate of membrane uptake of microcrystalline BP did not increase and the fluorescence emission spectrum did not change. Therefore only BP which has been adsorbed onto particles is more rapidly taken up into the membrane. The rate of BP uptake into phospholipid vesicles increases with decreasing surface density of BP on the particulates.

These results indicate that asbestos adsorbs BP in the monomeric form and is effective in transferring it to membranes. Crystalline BP is only slightly soluble in water. Therefore the co-carcinogenic effect of asbestos may be due, at least in part, to enhanced solubilization, transport and cellular uptake of BP.

Is hexachlorophene a carcinogen?

Rudali, G. & Assa, R. (1978). Lifespan carcinogenicity studies with hexachlorophene in mice and rats. *Cancer Lett.* **5**, 325.

Hexachlorophene (HCP) is now well-known for its effects on the central nervous system (*Cited in F.C.T.* 1977, **15**, 353; *ibid* 1979, **17**, 410). Some evidence of teratogenicity has also been obtained in rats and rabbits (*ibid* 1977, **15**, 355). However, a long-term mouse skin-painting test produced no indication of carcinogenicity (*ibid* 1976, **14**, 643) and negative results were also obtained in an NCI bioassay in which non-neurotoxic levels of 17, 50 or 150 ppm were included in the diet of rats for 105–106 wk (*Federal Register* 1978, **43**, 15785).

In the present study, two strains of mouse (groups of 68 C57Bl and 78 XVIII/G mice) were given 150 ppm HCP in a complete diet for 2 yr, and 15 Sprague–Dawley rats were given 50 ppm HCP in a protein- and vitamin-deficient diet for a similar period. HCP (0·5 mg) was also administered daily for 20 days from parturition to seven lactating mice, the 41 offspring of which were maintained for their lifespan. Other groups of 52 mice were similarly maintained either after receiving three sc injections of 0·05,

0·05 and 0·1 mg HCP on days 1, 2 and 8 of life, or after their birth to females that had been injected with 0·5 mg HCP on days 16 and 18 of pregnancy.

No neurological or other obvious toxic effects were observed in any of these animals, and body weights and lifespan were unaffected. Tumour incidence was not significantly increased in any group, although three hepatomas, which are very rare in the XVII/G strain used, were found in mice that had received HCP via the mother's milk. One pituitary adenoma also occurred in a 103-wk-old rat, but this was regarded as of spontaneous origin. It was concluded that the study had produced no convincing evidence of carcinogenicity.

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LETTER TO THE EDITOR

MUTAGENICITY TESTING OF THE URINE OF RATS TREATED WITH AMARANTH*

Sir,—Since Baigusheva (*Vop. Pitan.* 1968, **27**, 46) found sarcomas of the peritoneum and intestine in rats after oral intubation of amaranth paste, the dye has been reinvestigated repeatedly in respect of its potentially carcinogenic and mutagenic effects.

With regard to the mutation-based *in vitro* tests, amaranth showed no activity in the Salmonella/microsome mutagenicity assay (e.g. Lecointe & Lesca, *Fd Cosmet. Toxicol.* 1978, **16**, 89). However, in the fluctuation test using *Salmonella typhimurium* strain TA1538 and *Escherichia coli* WP2 as test organism, amaranth proved positive (Gatehouse, *Mutation Res.* 1978, **53**, 289). With the *S. typhimurium* strains G46 and TA1530, the dye similarly evoked a mutagenic response in the host-mediated assay, and it was suggested that this mutagenic response was induced by some metabolite (Joint FAO/WHO Expert Committee on Food Additives, WHO Fd Add. Ser. no. 8 (p. 15), Geneva, 1975). Radomski & Mellinger (*J. Pharmac. exp. Ther.* 1962, **136**, 259) demonstrated that amaranth undergoes reductive cleavage in the gut, by the intestinal flora, and that the reaction products are absorbed from the intestine. Mutagenicity testing of amaranth should therefore be supplemented with *in vivo* tests, taking account of the possible effects of such metabolites. Therefore, we investigated the mutagenic potency of urine samples from rats treated with amaranth, using the Ames test. Since urinary metabolites are frequently eliminated as glucuronide conjugates, plates containing β-glucuronidase were also run, with and without microsomal activation.

Amaranth was obtained from Merck AG (Darmstadt) and β-glucuronidase type V-A from Sigma (München). The following strains of *S. typhimurium* were used: TA100 and TA1535, which are sensitive to mutagens causing base-pair substitutions, and TA98 and TA1538, which are sensitive to mutagens causing frameshift mutations. Manipulation of the tester strains and preparation of the rat-liver homogenate fraction 'S-9' and an 'S-9 mix' (containing 0·1 ml S-9 fraction/ml of mix) were carried out as described by Ames *et al.* (*Mutation Res.* 1975, **31**, 347). Livers from male Sprague-Dawley rats of 180–200 g were used, and 5 days before they were killed the animals were injected ip with a corn-oil solution of Aroclor 1254 (200 mg/ml) to give a dose of 500 mg/kg body weight.

Urine was tested both by the standard plate incorporation assay described by Ames *et al.* (*loc. cit.*) and by a modification of that method, urine (0·1 or 0·2 ml), tester bacteria (0·1 ml), S-9 mix (0·5 ml, if required) and β-glucuronidase (670 units, if required) being pre-incubated at 30°C for 2 hr before addition to the top agar and plating. The control mutagens were sodium azide and 2-aminofluorene. All experiments were repeated two or three times with and without S-9 mix; plates with β-glucuronidase were also run with and without microsomal activation.

In view of possible age-related differences in intestinal enzyme activities, male Sprague-Dawley rats weighing 120 ± 10 g and 300 ± 20 g were used in these studies. The rats were fed a standard diet (Herilan-Zucht Pellets from Eggersmann KG, D-3260 Rinteln). After amaranth administration, the animals were housed individually in metabolism cages. Urine was collected in chilled tubes. Each of the four experimental groups consisted of 4–6 animals of the two weight classes.

Aqueous solutions of amaranth were administered by stomach tube to animals of groups I and II. Each animal of group I received a single dose (100 mg amaranth/animal), while those of group II received two daily doses (2×100 mg/animal). Group III was injected ip with a single dose of 200 mg amaranth/kg body weight) and group IV served as the untreated control. The urine samples for the periods 0–24 and 24–48 hr after amaranth treatment were collected and, after sterile-filtration, were assayed separately by the mutagenicity test.

In none of the experimental groups were signs of induced revertants observed. All plate counts were in the range of the spontaneous mutation rate (21 ± 5 for TA 1535, 109 ± 16 for TA100, 15 ± 5 for TA1538 and 33 ± 7 for TA98). High mutation rates were observed with the positive controls, sodium azide and 2-aminofluorene.

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ANNOUNCEMENT

TOXICOLOGY SOCIETY FORMED IN INDIA

A group of leading Indian scientists has recently formed the Society of Toxicology, India, to foster the progress and development of all aspects of toxicology. Those wishing to join the Society should contact Dr. C. R. Krishnamurti, Director of the Industrial Toxicology Research Centre, Post Box 80, Lucknow-226001, or Dr. P. K. Gupta, Convener of the Society. The secretariat of the Society of Toxicology, India is located at the Industrial Toxicology Research Centre.

Fd Cosmet. Toxicol. Vol. 17, p. 567. Pergamon Press Ltd 1979. Printed in Great Britain

FORTHCOMING PAPERS

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

Analysis of volatile *N*-nitrosamines in alcoholic beverages. By E. U. Goff and D. H. Fine.

Mutagenicity of Chinese alcoholic spirits. By J. S. K. Lee and L. Y. Y. Fong.

Formation d'un cancérigène, le *N*-nitrosocarbaryl, par interactions entre un insecticide de la série des carbamates, le carbaryl, et le nitrite de sodium dans le suc gastrique de rat. Par M. Beraud, B. Pipy, R. Derache et D. Gaillard.

Lysinoalanine formation in alkali-treated proteins and model peptides. By N. I. Karayiannis, J. T. MacGregor and L. F. Bjeldanes.

Biological effects of alkali-treated soya protein and lactalbumin in the rat and mouse. By N. I. Karayiannis, J. T. MacGregor and L. F. Bjeldanes.

Acute toxicity of patulin and its interaction with penicillic acid in dogs. By C. S. Reddy, P. K. Chan, A. W. Hayes, W. L. Williams and A. Ciegler.

Quantitative and morphological aspects of cutaneous irritation by trichothecene mycotoxins. By M. A. Hayes and H. B. Schiefer.

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

Effects of cadaverine on histamine transport and metabolism in isolated gut sections of the guinea-pig. By H.-Y. Paik Jung and L. F. Bjeldanes.

Percutaneous absorption of dodecyltrimethylammonium bromide, a cationic surfactant, in the rat. By F. G. Bartnik and F. Wingen.

Reproduction and teratology studies of zinc pyrithione administered orally or topically to rats and rabbits. By G. A. Nolen and T. A. Dierckman.

Neurological, microscopic and enzyme-histochemical assessment of zinc pyrithione toxicity. By D. R. Snyder, C. P. V. de Jesus, J. Towfighi, R. O. Jacoby and J. H. Wedig.

Toxicology studies, II. The laboratory animal. By J. G. Fox, P. Thibert, D. L. Arnold, D. R. Krewski and H. C. Grice. (Review Paper)