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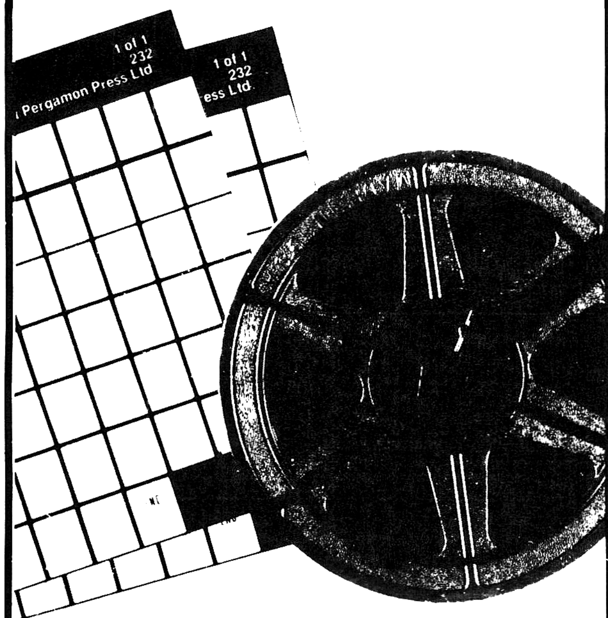

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Research Section

THE METABOLIC DISPOSITION OF ^{14}C-LABELLED PONCEAU 4R IN THE RAT, MOUSE AND GUINEA-PIG

J. C. PHILLIPS, C. S. BEX and I. F. GAUNT

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(*Received 18 March 1982*)

Abstract—The absorption, metabolism and excretion of ^{14}C-labelled Ponceau 4R has been studied in the rat, mouse and guinea-pig. Following administration of a single oral dose of 0·5 or 50 mg/kg body weight substantially all of the dose was excreted in the urine and faeces within 72 hr, with the majority being accounted for in the faeces. In all three species, naphthionic acid was the major urinary metabolite, whereas in the faeces naphthionic acid, 7-hydroxy-8-aminonaphthalene-1,3-disulphonic acid and unchanged dye were found. Pretreating male rats with unlabelled Ponceau 4R in the diet (50 mg/kg/day) for 28 days prior to dosing with the ^{14}C-labelled colouring had no effect on the route of excretion or the time taken to eliminate the majority of the label. Following a single dose of ^{14}C-labelled colouring to previously untreated rats, mice and guinea-pigs or to rats pretreated as above, no marked accumulation of radioactivity in any tissue was found, although tissue levels of radioactivity at 72 hr after dosing were higher in the pretreated rats than in those that were not pretreated. Pregnant rats eliminated a single oral dose of ^{14}C-labelled colouring at a similar rate to non-pregnant females; however, some retention of radioactivity in the foetuses was found. In studies of absorption from isolated loops of small intestine containing 50, 500 or 5000 ppm Ponceau 4R, no significant absorption was detected in rats, but some absorption was seen in mice at the lowest concentration, and in the guinea-pig at the two higher concentrations.

INTRODUCTION

The principal constituent of Ponceau 4R [CI (1971) No. 16255; EEC E.124] is the trisodium salt of 7-hydroxy-8-(4-sulphonato-1-naphthylazo)naphthalene-1,3-disulphonate. It is one of the water-soluble azo dyes currently permitted for food use in the United Kingdom (Food Additives and Contaminants Committee, 1979).

A considerable body of evidence relating to the toxicology of azo dyes is available (IARC Working Group, 1975; Joint FAO/WHO Expert Committee on Food Additives, 1974; Khera & Munro, 1979), and the metabolism of these compounds has been reviewed (Albrecht & Manchon, 1973; Walker, 1970). It has been shown that for most sulphonated azo-dyes, the majority of an orally administered dose is metabolized by reductive fission of the azo group, and that only a small proportion is absorbed or excreted intact (Larsen & Tarding, 1976; Radomski & Mellinger, 1962; Urakubo, 1967). No information is available on the metabolic fate of Ponceau 4R in the intact animal; however, it has been demonstrated that this dye is subject to azo-reductive fission by rat caecal contents *in vitro* (Larsen, Meyer & Scheline, 1976; Walker, 1968) and during fermentation by lactic acid bacteria (Eisenbrand & Lohrscheid, 1959).

The results presented in this report describe the absorption, distribution and excretion of ^{14}C-labelled Ponceau 4R in the rat, mouse and guinea-pig following oral administration at dose levels of 0·5 and 50 mg/kg body weight.

Abbreviations: 7-HANS = 7-Hydroxy-8-aminonaphthalene-1,3-disulphonic acid.

EXPERIMENTAL

Materials. Ponceau 4R labelled with ^{14}C in both naphthalene rings (1,4,5,8,1′,4′,5′,8′-^{14}C; Fig. 1) was supplied by ICI Physics and Radioisotope Services, Cleveland. It had a specific activity of 31·8 mCi/mmol. Thin-layer chromatography of the labelled material on silica-gel plates developed in propan-2-ol–ammonia (sp. gr. 0·88)–water (10:1:2, by vol.; system 1) or butan-1-ol–propan-1-ol–ammonia (sp. gr. 0·88)–water (10:5:1:4, by vol.; system 2) or on cellulose plates developed in butan-1-ol–ethanol–water (5:2:3, by vol.; system 3) revealed one red component (R_F in the three systems 0·30, 0·22 and 0·37, respectively). The radiochemical purity of the material in all three chromatographic systems was >90%.

Unlabelled Ponceau 4R (Ref. 9/1422N) was supplied by the Food Colours Sub-Committee of the Chemical Industries Association, and complied with the specifications laid down in the Food Additives and Contaminants Committee (1979) report. Analysis by Williams (Hounslow) Ltd, Hounslow, Middx showed that the material contained: dye, 81%; subsidiary dyes, 0·5%; volatile matter, 5·8%; water insoluble matter, 0·02%; sodium chloride, 7·9%; sodium sulphate, 0·9%; copper, 2 ppm; lead, 5 ppm; arsenic, 1 ppm. Thin-layer chromatography of this material in solvent systems 1, 2 and 3 revealed only one coloured component with an R_F value similar to that of the labelled colour (R_F, 0·32, 0·23 and 0·40, respectively).

Animals and treatment. The studies were carried out in male and female Wistar albino rats (100–180 g body weight), pregnant female Wistar rats (200–250 g body weight) and male Dunkin–Hartley guinea-pigs

(210–360 g body weight), supplied by OLAC (1976) Ltd, Bicester, Oxon. The pregnant rats were supplied with a known day of mating (defined as day 0 of pregnancy). Male CD-1 mice were supplied by Charles River UK Ltd, Margate, Kent. The rats and mice were fed Spratt's Laboratory Animal Diet No. 1 and the guinea-pigs were fed Oxoid Diet SG1 with a vitamin C supplement in the drinking-water. All animals were given food and water *ad lib.* and kept at 20 ± 2°C.

Groups of male rats, mice and guinea-pigs and female rats were given a single dose of ^{14}C-labelled Ponceau 4R by gavage. The labelled colouring, diluted with unlabelled colouring, was administered as an aqueous solution at a volume (5 ml/kg body weight) that provided a dose level of 0·5 or 50 mg colouring/kg body weight (20 μCi/kg body weight). Pregnant rats were given a single oral dose of 50 mg ^{14}C-labelled Ponceau 4R/kg body weight on day 8 of pregnancy. In addition, a group of male rats was given unlabelled Ponceau 4R in the diet for 28 days to provide an intake of approximately 50 mg/kg body weight/day. This was followed by a single oral dose of 50 mg ^{14}C-labelled Ponceau 4R/kg.

After treatment with ^{14}C-labelled colouring the animals were housed in all-glass metabolism cages (Jencons Scientific Ltd, Hemel Hempstead, Herts). Air was drawn through the cage at a constant rate of 250 ml/min and the exhaled CO_2 was trapped in ethanolamine–2-ethoxyethanol (1:4, v/v). The trapping solutions were changed at intervals up to 72 hr. Urine and faeces were collected at 24-hr intervals for 3 days, after which time the animals were killed.

The liver, kidneys, spleen, heart, lungs, brain, gastro-intestinal tract (including contents), testes (genital tract in non-pregnant females), foetuses and samples of skeletal muscle and subcutaneous fat were analysed for radioactivity.

Urinary excretion. Urine samples (0–24 hr) were lyophilized and the residue extracted repeatedly with methanol. The total methanolic extract was evaporated to dryness, taken up in methanol and examined by thin-layer chromatography. The residue from the methanol extraction was further extracted with ethyl acetate and the residue from this extraction dissolved in water. The ethyl acetate extract and the aqueous solution were also examined by thin-layer chromatography. For all three species, more than 80% of the radioactivity present in the urine was recovered in the methanol extract.

Faecal excretion. Faeces (5 g) were homogenized in 50 ml methanol–water (95:5, v/v), filtered and the residue repeatedly extracted with 25-ml aliquots of methanol until no further radioactivity could be removed. The combined methanol extracts were concentrated and examined by thin-layer chromatography. The radioactivity remaining in the residue was determined by combustion. For all three species more than 80% of the radioactivity present in the faeces was extracted by this procedure.

Biliary excretion. Three male rats were anaesthetized with sodium pentobarbitone and the bile duct of each was cannulated. The ^{14}C-labelled colouring dissolved in 0·9% (w/v) saline was injected into the stomach at a dose rate of 0·5 ml/100 g body weight to provide a dose level of 50 mg/kg. Bile was collected at intervals for up to 5 hr. The radioactivity excreted in the bile at each time interval was determined.

Intestinal absorption studies. Absorption was studied using the techniques described by Matthews, Craft, Geddes *et al.* (1968). Male animals of the three species were anaesthetized with sodium pentobarbitone and a length of small intestine was isolated between ligatures while ensuring that the mesenteric blood supply remained intact. Three separate 5-cm lengths were isolated with this length (only one for the mice) and 0·3 ml of a 0·9% (w/v) saline solution containing ^{14}C-labelled and unlabelled colouring giving a total concentration of colouring of either 50, 500 or 5000 ppm was introduced into each loop. Loops were removed immediately after injection of the sample (time = 0 min), after 30 min and after 1 hr and washed out with 0·9% (w/v) saline solution. The intestine and the contents were assayed separately for radioactivity.

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

Statistical analysis. Differences in the total excretion of radioactivity following a single oral dose of ^{14}C-labelled colouring were investigated by two-way analysis of variance. Intestinal absorption data were subjected to a Dixon's Gap test for outliers and absorption after 30 and 60 min was compared with zero-time controls using Student's t test. The level of significance chosen for all studies was $P < 0·05$.

RESULTS

The excretion of radioactivity by male and female rats following a single oral dose of ^{14}C-labelled Ponceau 4R is shown in Table 1. Although the majority of the radioactivity was eliminated in the faeces within 24 hr, between approximately 5 and 23% of the administered radioactivity was found in the urine during the 3-day collection period. Only trace quantities of radioactivity (less than 0·03% of the dose) were detected in expired CO_2. Male mice and guinea-pigs (Table 2) excreted a higher proportion of the dose in the urine (9–27% in the mouse and 9–37% in the guinea-pig) but these levels were not statistically significantly different from those for the rats.

Treating male rats with unlabelled Ponceau 4R in the diet for 28 days prior to dosing with ^{14}C-labelled colouring had no effect on the rate of excretion of the 50-mg/kg dose or on the proportion of the radioactivity excreted in urine and faeces (Table 1). The excretion of radioactivity in urine and faeces by pregnant rats given a single oral dose of ^{14}C-labelled

Table 1. *Excretion of radioactivity by male and female rats given a single oral dose of* [14]*C-labelled Ponceau 4R*

Route of excretion	Time (hr)	Dose (mg/kg)... 0·5	50	50*
		Males		
CO_2	0–24	0·001 (0·001–0·002)	0·001 (0·001–0·003)	0·007 (0·004–0·012)
	24–72	0·002 (all)	0·003 (0·002–0·006)	0·005 (0·002–0·008)
Urine	0–24	6·3 (4·3–10·0)	9·3 (6·1–12·2)	7·6 (5·1–10·1)
	24–48	0·7 (0·3–1·3)	1·5 (1·1–2·3)	1·3 (0·2–3·6)
	48–72	0·1 (0·1–0·2)	0·2 (0·1–0·2)	0·2 (0·04–0·55)
Faeces	0–24	87·6 (80·9–95·0)	78·4 (70·9–84·1)	75·9 (49·6–91·6)
	24–48	3·9 (1·4–6·7)	11·7 (9·3–14·4)	13·8 (3·7–36·3)
	48–72	0·3 (0·1–0·4)	0·6 (0·2–1·0)	1·3 (0·4–2·8)
		Total... 98·9 (97·0–101·4)	101·6 (94·1–106·9)	100·1 (94·0–103·2)
		Females		
CO_2	0–24	0·012 (0·011–0·012)	0·002 (0·001–0·003)	ND
	24–72	0·017 (0·010–0·028)	0·002 (all)	ND
Urine	0–24	7·9 (4·5–10·2)	8·9 (4·4–15·1)	8·9 (5·8–14·0)
	24–48	0·9 (0·4–1·9)	2·1 (0·5–7·0)	0·8 (0·2–1·4)
	48–72	0·1 (0·1–0·2)	0·3 (0·1–0·8)	0·3 (0·05–1·2)
Faeces	0–24	75·9 (58·9–85·2)	74·8 (31·8–91·1)	76·1 (49·1–92·1)
	24–48	11·9 (3·0–27·0)	12·8 (4·2–37·7)	14·8 (3·6–35·2)
	48–72	0·6 (0·3–1·2)	2·9 (0·3–10·3)	0·8 (0·3–2·6)
		Total... 97.4 (95·2–99·1)	101·8 (99·0–105·6)	101·7 (95·2–104·6)

Recovery of administered radioactivity (%)

ND = Not determined

*Male rats: pretreated for 28 days with unlabelled Ponceau 4R (*c.* 50 mg/kg body weight/day) given in the diet. Female rats: pregnant animals given a single oral dose on day 8 of pregnancy.

Values are means for groups of four low- and high-dose males and low-dose females and for groups of six high-dose females, pretreated male and pregnant rats. The range of values is given in brackets.

colouring was similar to that by non-pregnant females (Table 1).

The radioactivity recovered in the tissues of male and female rats is shown in Table 3. The proportion and distribution of the retained radioactivity was similar to both dose levels with the majority being associated with the gastro-intestinal tract, liver and kidneys. In female rats, the proportion of the dose retained was higher, particularly in the liver and gastro-intestinal tract (Table 3). Substantially higher levels of radioactivity were found in the tissues of pretreated rats although the distribution remained unchanged (Table 3). The proportion of the dose retained in the majority of the tissues was comparable in pregnant and non-pregnant animals (Table 3), although the livers of the pregnant animals contained substantially more radioactivity than those of the non-pregnant animals. Between 0·3 and 0·5% of the administered dose was present in the foetuses.

The tissue levels of radioactivity in male mice given ^{14}C-labelled Ponceau 4R at a dose level of 50 mg/kg were similar to those in the rat; however at the lower dose level the proportion of radioactivity retained was greater (Table 4). The male guinea-pig also retained a

Table 2. *Excretion of radioactivity by male mice and guinea-pigs given a single oral dose of* [14]*C-labelled Ponceau 4R*

Route of excretion	Time (hr)	Dose (mg/kg)...	Mouse 0·5	Mouse 50	Guinea-pig 0·5	Guinea-pig 50
CO_2	0–24		0·010 (0·004–0·016)	0·003 (0·001–0·004)	0·002 (0·001–0·004)	0·008 (0·001–0·026)
	24–72		0·004 (0·002–0·009)	0·002 (0·002–0·003)	0·002 (0·002–0·002)	0·004 (0·002–0·013)
Urine	0–24		13·7 (7·7–23·2)	11·8 (9·7–16·8)	7·8 (6·9–8·4)	14·0 (6·7–31·0)
	24–48		1·2 (0·7–2·8)	2·1 (1·2–5·3)	4·2 (2·2–6·2)	2·6 (1·5–5·5)
	48–72		0·4 (0·2–0·7)	0·4 (0·1–0·9)	0·9 (0·5–1·3)	0·3 (0·1–0·7)
Faeces	0–24		82·6 (61·4–94·9)	75·3 (60·1–86·3)	51·2 (36·9–60·6)	60·7 (33·8–75·2)
	24–48		2·0 (0·6–5·0)	7·9 (2·1–22·2)	28·4 (23·9–32·3)	18·2 (11·7–20·5)
	48–72		0·4 (0·1–0·7)	0·5 (0·3–1·1)	9·3 (5·0–17·3)	10·2 (8·6–13·8)
		Total...	100·3 (93·7–104·4)	98·0 (90·1–103·7)	101·8 (100·9–102·3)	106·0 (88·6–112·0)

Recovery of administered radioactivity (%)

Results are expressed as the mean for groups of six animals (three for guinea-pigs given 0·5 mg/kg), with the range of values in brackets.

Table 3. *Radioactivity in the tissues of male and female rats 72 hr after an oral dose of ^{14}C-labelled Ponceau 4R*

Tissue	Dose level (mg/kg)...	Recovery of administered dose (% × 10^3)					
		Male			Female		
		0·5	50	50*	0·5	50	50†
Liver		12·3 ± 1·9	10·0 ± 0·9	288 ± 15	84 ± 36	57 ± 8	497 ± 74
Kidney		7·4 ± 0·9	7·4 ± 0·7	20·2 ± 2·6	8·6 ± 1·6	9·2 ± 3·2	11·5 ± 0·6
Spleen		0·4 ± 0·03	0·5 ± 0·1	7·8 ± 3·9	1·3 ± 0·6	1·2 ± 0·4	3·1 ± 1·0
Brain		0·4 ± 0·03	0·3 ± 0·1	6·8 ± 0·3	4·9 ± 2·5	1·2 ± 0·4	4·6 ± 0·5
Heart		0·5 ± 0·2	0·3 ± 0·1	5·8 ± 0·6	0·6 ± 0·3	0·9 ± 0·3	3·7 ± 0·3
Testes/genital tract		0·8 ± 0·1	1·0 ± 0·1	13·0 ± 0·9	1·0 ± 0·5	0·9 ± 0·2	ND
Lungs		0·8 ± 0·2	0·7 ± 0·1	9·0 ± 1·5	0·8 ± 0·3	3·1 ± 2·0	5·3 ± 0·7
Stomach		1·6 ± 0·4	3·1 ± 0·3	7·6 ± 1·4	3·9 ± 1·8	13·2 ± 6·7	9·3 ± 1·8
Small intestine		14·0 ± 2·2	20·5 ± 1·7	362 ± 57	38 ± 6	133 ± 50	253 ± 22
Caecum		8·3 ± 0·6	14·0 ± 4·4	53 ± 21	56 ± 33	77 ± 47	189 ± 41
Large intestine		11·3 ± 2·6	15·0 ± 2·7	51 ± 26	32 ± 10	58 ± 39	10·8 ± 1·1
Muscle‡		0·5 ± 0·1	0·3 ± 0·1	4·9 ± 0·9	0·2 ± 0·1	1·6 ± 0·3	3·8 ± 0·4
Fat‡		2·0 ± 0·7	2·4 ± 0·4	19·4 ± 3·7	5·6 ± 0·8	12·0 ± 1·6	22·8 ± 8·5

ND = Not determined

*Rats were pretreated for 28 days with unlabelled Ponceau 4R (*c.* 50 mg/kg body weight/day) given in the diet.
†Rats given a single oral dose on day 8 of gestation.
‡% of dose × 10^3/g tissue.
Values are means ± SEM for groups of four rats (six pretreated male rats and pregnant rats).

higher proportion of the administered radioactivity than the rat, although, as with the rat, there were no marked differences between the dose levels (Table 4). In the guinea-pig a substantial amount of the administered radioactivity remained in the gastro-intestinal tract (2·5–6% at the lower dose level and 1·5–4% at the higher dose level), and in the liver (0·3–1·4%) at 72 hr.

In the studies of intestinal absorption at concentrations of Ponceau 4R of 50, 500 or 5000 ppm no significant absorption of radioactivity (as measured by total recovery of injected radioactivity) from the rat small intestine occurred over a 1-hr period (Table 5). However, a slight, but statistically significant, loss of radioactivity after 1 hr was seen in the guinea-pig at the two higher concentrations (500 and 5000 ppm) and in the mouse at the lowest concentration (50 ppm). Although substantial, but variable, amounts of radioactivity were associated with the gut tissue, there was no relationship between the distribution of radioactivity in tissue and contents, with either time or concentration. The radioactivity associated with the gut tissue probably represents colouring bound to or associated with mucosal proteins. Measurement of the absorbance at 465 nm of a solution of the loop contents at the end of the experimental period showed that no breakdown of the azo dye had occurred. The lack of any substantial absorption of the intact azo dye from the upper gastro-intestinal tract of the rat was further supported by the observation that less

Table 4. *Radioactivity in the tissues of male mice and guinea-pigs 72 hr after an oral dose of ^{14}C-labelled Ponceau 4R*

Tissue	Dose level (mg/kg)...	Recovery of administered dose (% × 10^3)			
		Mouse		Guinea-pig	
		0·5	50	0·5	50
Liver		109 ± 39	4·0 ± 0·6	343 ± 37	536 ± 183
Kidney		62 ± 17	3·0 ± 1·0	16·0 ± 1·5	26·2 ± 4·4
Spleen		22 ± 7	2·2 ± 1·8	4·2 ± 2·0	8·7 ± 5·9
Heart		30 ± 12	0·5 ± 0·2	5·8 ± 1·9	7·1 ± 1·4
Lung		32 ± 14	0·5 ± 0·2	10·3 ± 1·3	12·6 ± 3·5
Brain		21 ± 7	0·4 ± 0·1	17·0 ± 1·5	22·0 ± 6·5
Testes		68 ± 36	1·7 ± 0·6	6·6 ± 0·5	6·8 ± 1·5
Stomach		38 ± 13	12·4 ± 4·1	595 ± 97	390 ± 58
Small intestine		90 ± 30	8·8 ± 1·8	318 ± 51	283 ± 26
Caecum		52 ± 10	16·4 ± 4·8	1941 ± 677	774 ± 202
Large intestine		74 ± 17	20·2 ± 4·0	1246 ± 277	599 ± 146
Muscle*		153 ± 23	20·0 ± 5·8	8·0 ± 0·6	25·8 ± 4·3
Fat*		199 ± 18	70 ± 14	8·0 ± 1·2	18·3 ± 1·8

*% of dose × 10^3/g tissue.
Values are means ± SEM for groups of six animals (three guinea-pigs given 0·5 mg/kg).

Table 5. *Absorption of ^{14}C-labelled Ponceau 4R from isolated loops of small intestine of male rats, mice and guinea-pigs*

Species	Time (hr)	Initial concn (ppm)... 50	500	5000
		Retained radioactivity (% of injected dose)		
Rat	0	102·8 ± 0·5 (7)	102·1 ± 1·3 (6)	99·2 ± 0·4 (3)
	0·5	104·4 ± 2·6 (6)	99·6 ± 2·4 (6)	99·5 ± 0·3 (5)
	1·0	101·4 ± 1·2 (6)	99·7 ± 4·0 (9)	96·3 ± 2·3 (12)
Mouse	0	103·5 ± 1·8 (7)	103·4 ± 0·6 (5)	103·5 ± 0·9 (3)
	0·5	104·4 ± 2·6 (6)	99·6 ± 2·4 (6)	99·5 ± 0·3 (5)
	1·0	101·4 ± 1·2 (6)	99·7 ± 4·0 (9)	96·3 ± 2·3 (12)
Guinea-pig	0	104·4 ± 1·2 (7)	104·0 ± 0·4 (3)	104·9 ± 0·3 (6)
	0·5	102·4 ± 3·0 (5)	102·2 ± 1·9 (6)	103·9 ± 4·6 (5)
	1·0	105·2 ± 2·7 (5)	100·4 ± 1·2 (3)*	98·7 ± 0·4 (3)***

Results are expressed as mean ± SEM for the number of determinations shown in brackets. Values marked with asterisks are significantly lower (Student's *t* test) than the corresponding zero-time values (* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$).

than 0·1% of the administered radioactivity in the 50-mg/kg dose was recovered in the bile after 1 hr and only between 0·6 and 1·3% was recovered after 5 hr.

At least two radioactive components were present in the urine of all three species. The major component (70% of the radioactivity in the rat) present in the methanol extract, co-chromatographed with naphthionic acid in three systems. Of the minor components, one had a similar chromatographic mobility to 7-hydroxy-8-aminonaphthalene-1,3-disulphonic acid (7-HANS; Fig. 2). In mouse urine, some radioactivity also co-chromatographed with Ponceau 4R. Thin-layer radiochromatography of faecal extracts from rats, mice and guinea-pigs given 50 mg ^{14}C-labelled Ponceau 4R/kg body weight showed that at least three radioactive compounds were present. Radioactivity in the extracts co-chromatographed with naphthionic acid, 7-HANS and unchanged Ponceau 4R.

DISCUSSION

Studies on the absorption and excretion in the rat of azo dyes with structures related to that of Ponceau 4R, such as amaranth (Honohan, Enderlin & Ryerson, 1976; Radomski & Mellinger, 1962; Ruddick, Craig, Stavric *et al.* 1979) and carmoisine (Galli & Costa, 1980) have shown that the majority of the administered dose is reduced in the gastro-intestinal tract, and that the metabolites are excreted preferentially in the faeces.

The results of our studies with ^{14}C-labelled Ponceau 4R in the rat showing that substantially all of a single oral dose is rapidly excreted in urine and faeces, with the majority accounted for in the faeces, suggests that the metabolic fate of Ponceau 4R in the rat is similar to that of related azo dyes. The presence of naphthionic acid in both urine and faeces confirms that this colouring is reduced in the gastro-intestinal tract of the rat.

The results obtained with mice and guinea-pigs suggest that the fate of Ponceau 4R in these species is similar to that in the rat. However, since both the mouse and the guinea-pig excreted a greater proportion of the dose in the urine than did the rat, there may be species differences in either the rate or extent of azo reduction in the gastro-intestinal tract or in the rate of absorption of the naphthionic acid released. The slower excretion of radioactivity in the faeces of the guinea-pig (approximately 10% of the dose is excreted between 48 and 72 hr compared with less than 1% in the male rat and mouse) is probably associated with a longer gut transit time in this species. Similar results have been reported with the essentially non-absorbed triphenylmethane dyes Green S and Brilliant Blue FCF in these three species (Phillips, Mendis, Eason & Gangolli, 1980).

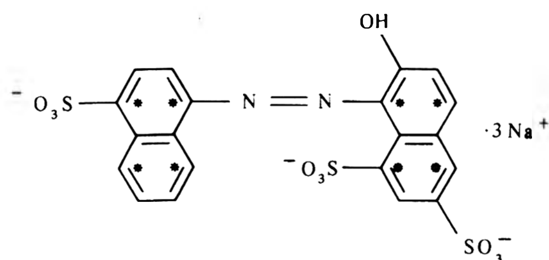

Fig. 1. Structure of Ponceau 4R with the ^{14}C-labelled positions indicated by an asterisk.

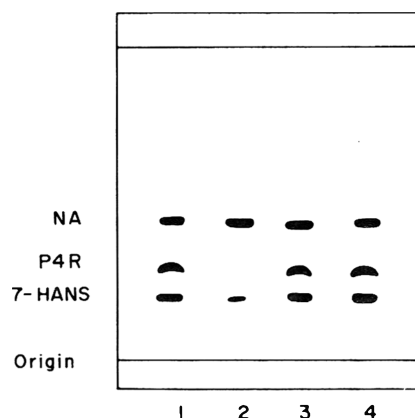

Fig. 2. Thin-layer radiochromatograms of urine extract (track 2) and faeces extracts (track 3) from rats given 50 mg ^{14}C-labelled Ponceau 4R/kg body weight. Standards (tracks 1 and 4) are Ponceau 4R (P4R), naphthionic acid (NA) and 7-hydroxy-8-aminonaphthalene-1,3-disulphonic acid (7-HANS).

The absorption and excretion of intact azo dye after oral administration has been reported for a number of colourings including amaranth (Ruddick *et al.* 1979). Ponceau SX and Sunset Yellow FCF (Radomski & Mellinger. 1962). In the present study, no evidence was found for absorption or for excretion in the urine of unchanged Ponceau 4R in the rat. However, chromatographic examination of 24-hr urine samples from mice revealed traces of unchanged Ponceau 4R. Although the gut-loop experiment suggested that there was significant absorption of Ponceau 4R from the mouse small intestine at the lowest concentration, this is not supported by the findings at the higher concentrations. In the light of this evidence it is unlikely that unchanged Ponceau 4R is absorbed from the upper gastro-intestinal tract of the mouse to any significant extent, and it is possible that the trace of dye found in mouse urine arises from contamination of urine by faeces during the experiment. Although there is evidence for absorption of radioactivity from guinea-pig gut at the higher concentrations, intact Ponceau 4R was not detected in the urine.

The low level of retention of Ponceau 4R and/or metabolites in tissues is similar to that of other related water-soluble azo dyes that have been investigated, including amaranth (Ruddick *et al.* 1979), Ponceau MX (Urakubo, 1967) and Sunset Yellow FCF (Honohan, Enderlin, Ryerson & Parkinson, 1977). Although the total residual radioactivity in most of the tissues was similar in male and female rats, that in the livers of the female rats was significantly greater than in the males. This may be of relevance to the finding that in both long-term (Allmark, Mannel & Grice, 1957) and short-term (Gaunt, Farmer, Grasso & Gangolli, 1967) toxicity studies on Ponceau 4R in this species, liver enlargement and increased serum transaminase levels have been reported in the female rat. Residual levels of radioactivity in the tissues of pregnant rats were low; however a substantial amount of radioactivity was found in the foetuses of these animals. Nevertheless no embryotoxic effects have been reported for Ponceau 4R in the rat at dose levels in excess of those used in this study (Meyer & Hansen. 1975).

The substantially higher tissue levels of radioactivity in the 28-day pretreated animals, suggests that the pharmacokinetics of a single dose of Ponceau 4R is altered by pretreatment. However, as the urinary excretion of radioactivity is unchanged, a marked increase in the extent of azo reduction is unlikely. Holson, Schumacher, Gaylor & Gaines (1976) have reported that pretreatment of rats with the related azo dye amaranth increases the capability of the gut flora to metabolize the colouring and hence increase absorption and excretion.

Thin-layer chromatography of extracts from the faeces of all three species suggested that some unchanged colouring was excreted. Studies on azo dyes with sulphonate groups adjacent to the azo link have generally shown that only trace quantities of an orally administered dose are excreted unchanged in the faeces (Radomski & Mellinger, 1962; Ryan & Welling, 1967; Scheline & Longberg, 1966). However the recent study with amaranth (Ruddick *et al.* 1979) showed that a substantial amount of unchanged dye was present in the faeces extract, although no quantitative data were reported.

Very little radioactivity (less than 1·5% of the dose) was found in the bile of rats given ^{14}C-labelled Ponceau 4R, indicating that biliary excretion of Ponceau or its metabolites is of minimal importance in this species. However, studies of the biliary excretion of water-soluble sulphonated azo dyes have shown that unchanged dye and metabolites are excreted in bile from animals given azo dyes including Ponceau 4R by both oral and parenteral routes (Walker, 1970) although Ikeda & Uesugi (1973) found no evidence for the biotransformation of Ponceau 4R after iv administration to bile cannulated, renal pedicle-ligated rats.

Further studies are in progress to characterize the metabolites of Ponceau 4R and to investigate possible species differences in the rate of azo reduction of the colouring.

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INFLUENCE OF GALLIC ACID ESTERS ON DRUG-METABOLIZING ENZYMES OF RAT LIVER

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Abstract—The effect of three antioxidants, propyl, octyl and dodecyl gallate, on hepatic drug metabolism in male rats was studied *in vivo* and *in vitro*. When fed at a dietary concentration of 1% for 14 days, only dodecyl gallate increased relative liver weight. Cytochrome *P*-450 content was not influenced, but a slight increase in cytochrome b_5 content was observed after the feeding of propyl gallate. Monooxygenase activity (benzo[*a*]pyrene-hydroxylase and ethoxycoumarin-deethylase activities) was not affected by propyl or octyl gallate, but a significant decrease in benzo[*a*]pyrene-hydroxylase activity was apparent in rats fed dodecyl gallate. Study of benzo[*a*]pyrene-metabolite formation in liver microsome preparations from control and propyl gallate-treated rats showed an overall decrease in metabolite production following gallate treatment, the decrease being statistically significant for the formation of the 9,10-dihydrodiol. Epoxide-hydratase activity was enhanced by a factor of 1·5 in rats fed propyl gallate; glutathione-transferase activity was unaffected. *In vitro*, the gallates proved to be potent inhibitors of ethoxycoumarin deethylation in liver microsomes from untreated and phenobarbital-treated rats; however, when cytochrome *P*-448 had been induced by pretreatment with 3-methylcholanthrene, ethoxycoumarin deethylase was less sensitive to the inhibitory action of the gallates.

INTRODUCTION

Antioxidants used in foods and feeds have been shown to affect drug-metabolizing enzymes in rodent tissues. Most of these studies have been performed with BHA, BHT and ethoxyquin, and it has been shown that these agents show both inducing and inhibiting properties towards enzymes involved in the biotransformation of foreign compounds. Liver microsomal monooxygenase can be induced by BHA and BHT (Allen & Engblom, 1972) as well as by ethoxyquin (Parke, Rahim & Walker, 1974a), and it has been demonstrated that the characteristics of this monooxygenase induction resemble those of phenobarbital induction (Kahl & Netter, 1977; Kahl & Wulff, 1979). Monooxygenase can be inhibited *in vitro* by these antioxidants (Hill & Shih, 1974; Parke, Rahim & Walker, 1974b; Yang, Strickhart & Woo, 1974), and the phenobarbital-inducible form of monooxygenase is far more sensitive to inhibition than is the 3-methylcholanthrene-inducible form (Kahl & Netter, 1977; Kahl & Wulff, 1979). Moreover enzymes that act on foreign compounds as a sequel to primary oxidation, i.e. epoxide hydratase (Cha, Martz & Bueding, 1978; Kahl, Deckers-Schmelzle & Klaus, 1978; Kahl & Wulff, 1979), glucuronosyl transferase (Bock, Kahl & Lilienblum, 1980; Cha & Bueding, 1979), glutathione transferase (Benson, Batzinger, Ou *et al.* 1978) and NADPH-quinone reductase (Benson, Hunkeler & Talalay, 1980), are also induced by these antioxidants.

In a number of countries, gallic acid esters are used as antioxidants in food and feed. However, the biological effects of this class of antioxidants have been less extensively characterized than those of BHA, BHT and ethoxyquin. Some studies have been performed with propyl and octyl gallate (Creaven, Davies & Williams, 1966; den Tonkelaar, Verschuuren, Kroes & van Esch, 1968). In one of these studies no increase in liver weight was caused by feeding octyl gallate at 0·5% to rats and no increase in biphenyl-4-hydroxylation was observed (Creaven *et al.* 1966). Recently King & McCay (1981) reported that the cytochrome *P*-450 content, monooxygenase activity and NADPH-cytochrome *c* reductase activity in rat-liver microsomes was not affected by feeding 0·3% propyl gallate. Inhibition of microsomal monooxygenase by propyl gallate has been observed *in vitro*, however, by Torrielli & Slater (1971) and by Yang & Strickhart (1974).

Data on the action of other gallic acid esters on monooxygenase are lacking. Moreover, other drug-metabolizing enzymes have not been tested for inducibility by gallic acid esters. The present paper deals with these questions and also characterizes the type of monooxygenase inhibition exerted by propyl gallate, octyl gallate and dodecyl gallate.

EXPERIMENTAL

Test compounds. Propyl gallate was purchased from Sigma Chemie AG, München, octyl and dodecyl gallates from Fluka AG, Neu-Ulm, and ethoxyquin from Paesel GmbH, Frankfurt.

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Abbreviations: BHA = Butylated hydroxyanisole; BHT = Butylated hydroxytoluene; HPLC = high-pressure liquid chromatography.

Animals and treatments. Groups of five male Sprague–Dawley rats (obtained from Wiga GmbH, Sulzfeld, and weighing 100 g at the start of the experiment) were fed for 14 days on a conventional powdered diet (Altromin 1320 from Altromin GmbH, Lippe) either unsupplemented (control group) or containing one of the gallates or ethoxyquin at a level of 1%. The rats were killed at the end of the feeding period, the livers were weighed and microsomal fractions were prepared. In addition to that brought about by the feeding of ethoxyquin, enzyme induction for *in vitro* studies was effected in male Sprague–Dawley rats weighing *c.* 200 g, either by three ip injections of 80 mg phenobarbital in normal saline given at 24-hr intervals or by three ip injections of 20 mg 3-methylcholanthrene in peanut oil given at 12-hr intervals. These rats were killed 24 hr after the last injection of phenobarbital and 36 hr after the last 3-methylcholanthrene injection. The effects of 5–500-μM concentrations of propyl, octyl or dodecyl gallate on ethoxycoumarin-deethylase activity were studied in preparations of liver microsomes from these rats.

Preparation of liver microsomes. Livers were weighed, perfused with ice-cold saline to remove erythrocytes and then homogenized with 3 vols 0·25 M-sucrose solution containing 0·02 M-Tris HCl buffer (pH 7·4), and 0·005 M-EDTA (Netter, 1960). Nuclear fragments and mitochondria were sedimented by centrifugation at 1600 g for 15 min and 9000 g for 30 min.

The microsomal pellet was obtained by centrifugation of the 9000-g supernatant at 105,000 g for 60 min. The pellet was washed once and then resuspended to a protein concentration of about 20 mg/ml. Protein content was determined according to Lowry, Rosebrough, Farr & Randall (1951). Spectral measurements were performed in a Perkin Elmer 356 two-wavelength double-beam spectrophotometer in the split-beam mode. The content of cytochrome *P*-450 and cytochrome b_5 was measured according to Omura & Sato (1964 a,b).

Benzo[*a*]pyrene-hydroxylase activity was determined by a modification of the fluorimetric procedure described by Gielen, Goujon & Nebert (1972). The assay was performed under subdued light. Ethoxycoumarin-deethylase activity was measured by a modification of the method described by Ullrich & Weber (1972). Epoxide-hydratase activity was measured with styrene oxide as the substrate according to Oesch, Jerina & Daly (1971) and with benzo[*a*]pyrene-4,5-oxide as the substrate according to Schmassmann, Glatt & Oesch (1976). [^{3}H]Styrene oxide and [^{3}H]benzo[*a*]pyrene-4,5-oxide were a generous gift from Professor F. Oesch, Mainz. Cytosolic glutathione *S*-transferase was measured with [^{3}H]benzo[*a*]pyrene-4,5-oxide as the substrate, as described by Van Cantfort, Manil, Gielen *et al.* (1979).

For studying benzo[*a*]pyrene metabolism using HPLC analysis, [^{3}H]benzo[*a*]pyrene (Amersham Buchler GmbH & Co. KG, Braunschweig) at a final specific activity of 0·2 Ci/mmol was incubated with liver microsomes for 15 min in a concentration of 50 μM. Extraction with ethyl acetate was followed by evaporation of the ethyl acetate phase and the residue was dissolved in methanol and injected into a Spectraphysics high-pressure liquid chromatograph (HPLC component system) equipped with a Spherisorb ODS 5 μ reverse-phase column. The elution was performed with a linear methanol/water gradient (35–75%) over 40 min at a constant flow of 0·5 ml/min. Between min 55 and 65, the gradient was increased to 100% methanol, and elution was then continued for another 10 min. Radioactivity was continuously monitored using an LB 503 monitor from Berthold, Wildbad. Data were stored for quantitation in the digital integration system BS 27/N (Berthold).

RESULTS

Table 1 summarizes the data on liver weight, microsomal cytochrome content and the activities of microsomal monooxygenase and epoxide hydratase and of cytosolic glutathione transferase obtained in feeding experiments with the gallates. A moderate increase in liver weight to body weight ratio was observed only after dodecyl gallate feeding. No influence of gallates on the cytochrome *P*-450 content of the microsomes was detected; cytochrome b_5 content tended to increase, and this increase was just statisti-

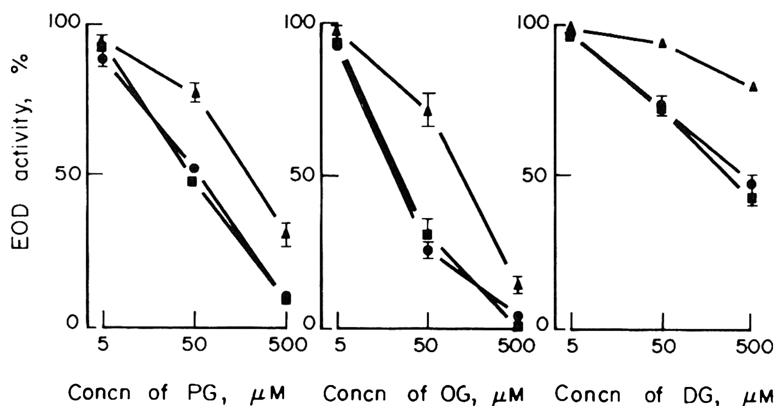

Fig. 1. Differential inhibition of ethoxycoumarin-deethylase activity *in vitro* by propyl gallate (PG), octyl gallate (OG) and dodecyl gallate (DG) added to liver microsomal preparations from untreated (●), phenobarbital-pretreated (■) and 3-methylcholanthrene-pretreated (▲) rats. Values are means ± SEM (n = 3).

Table 1. *Effect on liver weight and hepatic drug-metabolizing enzymes of feeding gallic acid esters to male rats at a dietary level of 1% for 14 days*

Parameter	Mean values			
Diet . . .	Control	Propyl gallate	Octyl gallate	Dodecyl gallate
Liver weight (g/100 g body weight)	4·31 ± 0·24	4·43 ± 0·19	4·39 ± 0·11	5·30 ± 0·22**
Cytochrome *P*-450 (nmol/mg protein)	0·85 ± 0·08	0·90 ± 0·05	0·82 ± 0·08	0·77 ± 0·09
Cytochrome b_5 (nmol/mg protein)	0·52 ± 0·02	0·65 ± 0·05*	0·61 ± 0·06	0·60 ± 0·06
Benzo[*a*]pyrene hydroxylase (pmol 3-hydroxybenzo[*a*]pyrene/min per mg protein)	241 ± 11	241 ± 20	230 ± 34	165 ± 12***
Ethoxycoumarin deethylase (nmol 7-hydroxycoumarin/min per mg protein)	2·00 ± 0·08	2·20 ± 0·18	2·00 ± 0·20	2·05 ± 0·15
Epoxide hydratase				
(nmol styrene glycol/min per mg protein)	11·00 ± 1·30	16·70 ± 1·40**	9·50 ± 1·00	12·60 ± 1·70
(nmol benzo[*a*]pyrene-4,5-dihydrodiol/min per mg protein)	3·61 ± 0·48	4·48 ± 0·15	3·32 ± 0·21	3·36 ± 0·30
Glutathione transferase (nmol benzo[*a*]pyrene-4,5-oxide-glutathione conjugate/min per mg protein)	7·11 ± 0·41	8·06 ± 0·42	8·14 ± 0·51	7·88 ± 0·36

Values are means ± SEM for groups of five rats and those marked with asterisks differ significantly (Student's *t* test) from the control values: $^*P = 0·05$; $^{**}P < 0·05$; $^{***}P < 0·005$.

cally significant with propyl gallate. While ethoxycoumarin-deethylase activity was not affected by gallate feeding, benzo[*a*]pyrene hydroxylase was inhibited by dodecyl gallate treatment, although it was unaffected by propyl- or octyl-gallate ingestion. Propyl gallate, but not the octyl or dodecyl ester, moderately increased microsomal epoxide-hydratase activity; the increase was statistically significant only when activity was measured with styrene oxide as the substrate. No effect on glutathione-transferase activity was observed.

Table 2, on the effect of propyl gallate feeding on the pattern of benzo[*a*]pyrene metabolism in liver microsomes, shows that this treatment impaired the rate of formation of all the dihydrodiols and phenols. Although this inhibition was statistically significant only for the production of the 9,10-dihydrodiol, a degree of inhibition close to statistical significance was apparent in the formation of the 4,5- and of the 7,8-dihydrodiol, preferentially mediated by cytochrome *P*-450 and cytochrome *P*-448, respectively (Rasmussen & Wang, 1974).

Figure 1 further illustrates the inhibitory action of gallates on microsomal monooxygenase activity, as demonstrated in *in vitro* experiments.

Microsomal preparations obtained from control and from phenobarbital-treated rats were more sensitive to the gallates than were those obtained from 3-methylcholanthrene-treated rats, which showed little response to inhibitor concentrations below 50 μM.

Table 3 demonstrates the *in vitro* effect of the gallates on epoxide hydratase. A tendency of all the gallates to activate this enzyme is apparent but is only statistically significant with octyl gallate and then only in microsomes in which epoxide-hydratase activity had been considerably induced by ethoxyquin feeding.

DISCUSSION

Dietary antioxidants are able to protect experimental animals against a wide spectrum of chemical carcinogens when they are fed prior to or concomitant with the administration of the carcinogen (for a review, see Wattenberg, 1978) Most of these studies have been performed with BHA and BHT and a few also with ethoxyquin. We know of only one study in which a gallic acid ester antioxidant has been shown to afford some protection against chemical carcinogenesis; King & McCay (1980) reported a reduction in dimethylbenz[*a*]anthracene-induced mammary tumorigenesis when rats were fed 0·3% propyl gallate in the diet. This seems surprising in view of the fact that gallic acid ester antioxidants play an important role as food additives in some countries, including the Federal Republic of Germany.

The mechanism underlying the protective action of antioxidants against carcinogens has not yet been elucidated. Most authors have considered the possibility of a relationship between this action and the effects of antioxidants on enzymes involved in the metabolism of the carcinogens. Inducing and inhibiting effects of BHA, BHT and ethoxyquin on drug metabolism have been widely studied, but only a few results on propyl gallate have been published, and none of the other

Table 2. *Pattern of benzo[a]pyrene metabolism by liver microsomes from rats fed powdered diet with or without a 1% propyl gallate supplement for 14 days*

Metabolite	Diet ...	Rate of metabolite formation (pmol/min per mg protein)	
		Control	PG
BP-9,10-dihydrodiol		70·4 ± 4·4	44·7 ± 7·5*
BP-4,5-dihydrodiol		62·2 ± 4·1	41·1 ± 9·1
BP-7,8-dihydrodiol		43·6 ± 2·4	26·7 ± 3·4
9-Hydroxy-BP		226·5 ± 68·4	140·9 ± 29·9
3-Hydroxy-BP		327·8 ± 87·5	261·0 ± 43·7

PG = Propyl gallate BP = Benzo[*a*]pyrene

Values are means ± SEM for groups of three or four determinations and that marked with an asterisk differs significantly ($P < 0{\cdot}05$ by Student's *t* test) from the control.

Table 3. *Effect of gallic acid esters* in vitro *on epoxide hydratase activity of liver microsomes from control rats and from rats fed 1% ethoxyquin in the diet for 14 days*

Treatment	Epoxide hydratase activity (nmol styrene glycol/min per mg protein)	
	Control microsomes	'Ethoxyquin' microsomes
Control	12·7 ± 2·2	97·3 ± 3·1
Propyl gallate (100 μM)	14·5 ± 2·4	102·4 ± 4·5
Octyl gallate (100 μM)	15·5 ± 2·8	127·6 ± 5·2*
Dodecyl gallate (100 μM)	13·9 ± 2·3	106·4 ± 6·5

Values are means ± SEM for three incubations and that marked with an asterisk differs significantly ($P < 0{\cdot}01$, by Student's *t* test) from the control.

gallates used as food antioxidants has been tested. No effects of propyl gallate on drug-metabolizing enzymes *in vivo* have been found in the studies published so far (Creaven *et al.* 1966; King & McCay, 1981) but *in vitro* inhibition of hepatic microsomal monooxygenase activity has been observed (Torrielli & Slater, 1971; Yang & Strickhart, 1974).

An absence of any *in vivo* effects of gallates on carcinogen metabolism would be an argument against the interpretation of their protective action against chemical carcinogenesis in terms of metabolic activation and inactivation, an interpretation favoured by a number of authors. Our study did not confirm prior indications that drug metabolism could not be affected by propyl gallate feeding, but the effects found were certainly very moderate, and it is questionable whether they are sufficient to provide support for that interpretation. Using 1% dietary levels of the antioxidants, we found inhibition of monooxygenase reactions by dodecyl gallate (benzo[*a*]pyrene-hydroxylase activity) and by propyl gallate (benzo[*a*]pyrene metabolite pattern) and induction of epoxide-hydratase activity by propyl gallate. It is not clear why dodecyl gallate proved the least potent monooxygenase inhibitor in the *in vitro* tests while *in vivo* it was the only gallate that was effective. Like ethoxyquin (Kahl & Netter, 1977) and BHA and BHT (Kahl & Wulff, 1979) the gallic acid esters were found to be preferential inhibitors of the constitutive and phenobarbital-inducible monooxygenase while cytochrome *P*-448-mediated monooxygenation was less sensitive, as was previously reported for propyl gallate (Yang & Strickhart, 1974).

This casts some doubt on the possible impact of monooxygenase inhibition as a critical mechanism in the anticarcinogenic action of the antioxidants because in many of the carcinogenesis test systems described cytochrome *P*-448 is likely to have been induced by the high dose of polycyclic aromatic hydrocarbon or other carcinogen administered. Our study provided no data to indicate whether antioxidant effects on one-electron flavoprotein-catalysed oxidations were likely to play a role in the anticarcinogenic activity.

Induction of epoxide hydratase may be assumed to be a protective mechanism against precarcinogens which form primary epoxides as the ultimate carcinogenic species. However, in the case of polycyclic aromatic hydrocarbons, including dimethylbenz[*a*]anthracene, which was used in the propyl gallate study of King & McCay (1980), the situation is different because not a primary oxide but a diol epoxide formed by the sequential action of monooxygenase, epoxide hydratase and again monooxygenase is the ultimate carcinogen. It is obvious that for diol-epoxide formation epoxide hydratase should play both an activating and a protective role. We have observed an increase in the formation of the DNA adduct of the diol epoxide of benzo[*a*]pyrene when metabolic activation of benzo[*a*]pyrene is catalysed by liver microsomes from ethoxyquin-treated rats (Kahl *et al.* 1978). However, ethoxyquin is a much more effective epoxide-hydratase inducer than is propyl gallate, as shown in the present study, and the biological significance of the moderate induction of the enzyme by the gallate remains questionable. *In vitro* data show that

the enhancement is not due to direct activation of the enzyme (Table 3). For ethoxyquin, it has been shown that the increase in enzyme activity is due to true enzyme induction (Kahl, 1980) and it remains to be tested whether this is also the case with propyl gallate.

The concentrations of gallates necessary to produce the effects on drug metabolism described here are far above those ingested by man in food. Even if it should turn out that the protection afforded by antioxidants against chemical carcinogenesis is due to intervention at the level of carcinogen metabolism, it does not seem likely that such protective action can also be expected from antioxidant supplementation of food. Moreover, since the mechanism of protection is still unclear and since some toxic effects of antioxidants are known—albeit at high dosage—an increase in antioxidant concentration in food does not appear to be justified, at least in the present state of knowledge.

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SUBCHRONIC TOXICITY STUDY IN RATS FED GUM KARAYA*

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Abstract—Gum karaya was given to groups of 15 rats of each sex at levels of 0 (control), 0·2, 1 or 5% (w/w) in the diet for 13 wk. An increase in faecal bulk was seen in all treated groups throughout the study. There was a decrease in weight-gain at the highest dietary level (significant only in the females), which was associated with a marginal reduction in food-conversion efficiency. Males given 1 or 5% drank more than the controls and a transient increase in water intake was seen in females given the highest level. The no-untoward-effect level from this study was 5% (w/w) of the diet, providing a mean intake of about 4 g karaya gum/kg body weight/day.

INTRODUCTION

Gum Karaya (Sterculia gum), the dried exudate from *Sterculia urens* Roxburgh and other Sterculia species (Fam. Sterculiaceae) is a partially acetylated polysaccharide of very high molecular weight. Structural studies (Aspinall & Sanderson, 1970) do not permit a complete structure to be proposed for any of the few species studied to date, but it appears that Sterculia gum molecules are highly branched, with interior galacturonorhamnan chains to which are attached galactose and rhamnose end-groups.

Because of its unusual stability towards acidic hydrolysis and enzymatic degradation, gum karaya has a long history of use at the 0·002 to 0·8% level in foodstuffs, pharmaceuticals and cosmetics as an emulsifier, stabilizer, thickener and bulking agent, particularly in acidic preparations.

Although the teratogenicity and mutagenicity of gum karaya have been investigated in studies commissioned by the US Food and Drug Administration (NTIS reports 1972, 1973), no satisfactory metabolic, 90-day or long-term studies with karaya have previously been reported. Since there was insufficient evidence to allow the establishment of an acceptable daily intake (ADI) gum karaya was included in Annex II of the EEC Directive on emulsifiers, stabilizers, thickeners and gelling agents (74/329/EEC as amended by 78/612/EEC) allowing it to be authorized temporarily by individual member states. In its 1978 report the Scientific Committee for Food declared that it would be prepared to re-assess the classification of gum karaya if the results of a metabolic and a 90-day study became available within one year. Consequently, small-scale preliminary dietary and dose-ranging studies were started in 1979 at Edinburgh University and application was made to the SCF by the sponsor of the present study (INGAR) for an extension until the end of 1980, of the time by which the necessary studies were to be completed and assessed. This extension was granted on 29 April 1980, in the second amendment (80/597/EEC) to Directive 74/329/EEC. Reports of the present study and of a metabolic study of gum karaya in rats (sponsored by INGAR) have been submitted to the SCF and a metabolic study in man was carried out early in 1982 to meet recommendations made by WHO (1974).

The preliminary dietary studies indicated a marked unpalatability at dietary levels of more than 8% gum karaya, presumably because of its odour of free acetic acid. Rats could be induced to eat diets containing more than 8% gum karaya by incremental increases in dietary concentrations, but at these higher levels (up to 16%) body-weight gain was considerably reduced. However, limited laboratory investigations and macroscopic *post mortem* examination revealed no adverse effects in animals that had received up to 16% gum karaya for a period of 90 days. A dietary concentration of 5% was selected as the highest dose level for the study reported here to avoid these problems of palatability and possible dietary imbalances.

EXPERIMENTAL

Test material. Gum karaya was obtained by a London importer in 1979 as a parcel of lumps (Batch No. 799) from an established Bombay shipper. The material was reduced commercially into powder (to pass through 150 mesh) and was then sifted on a 200 mesh sieve to remove ultra-fine powder. A sample of this batch was identified by Allport Consulting Analysts Ltd, London SE11 as gum karaya satisfying the requirements of the British Pharmaceutical Codex 1973. Other analyses showed the gum to be com-

*This investigation was sponsored by the International Natural Gums Association for Research (INGAR) Ltd, Marlon House, 71–74 Mark Lane, London EC3R 7HS.

Abbreviations: INGAR = International Natural Gums Association for Research Ltd; SCF = Scientific Committee for Food.

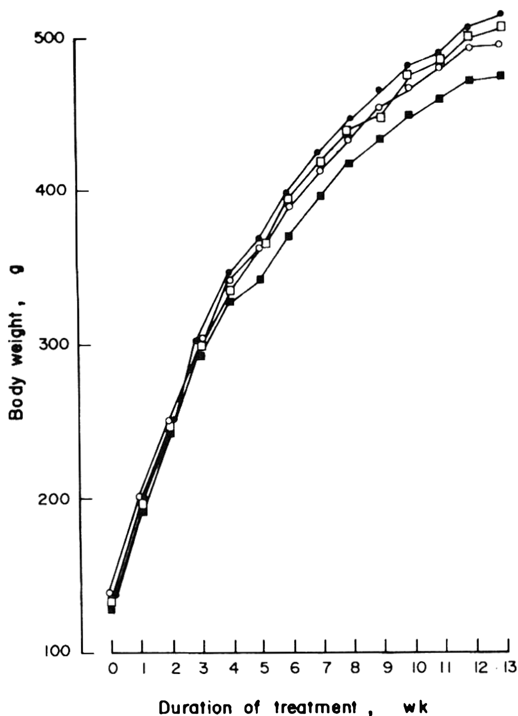

Fig. 1. Mean body weights of male rats fed diets containing 0(○) 0·2(●), 1(□) or 5(■)% gum karaya for 13 wk.

prised of moisture (17%), acetyl groups (14%), inorganic matter (7%) and carbohydrate (62%).

Animals. Sprague–Dawley derived rats from the CD strain (60 males, 60 females: 22 days old) were obtained from Charles River (UK) Ltd, Margate, Kent. They were housed in groups of five, by sex, in stainless-steel wire-mesh cages in environmentally controlled quarters (18–25°C, relative humidity 40–70%) with 12-hr light/dark cycles. Ground diet and tap water were freely available, except during periods of deprivation associated with laboratory investigations.

Preparation of test diets. The basic diet used was CRM-X, GLP-certified (Labsure, Poole, Dorset). Batches of diet were prepared for each treatment group at fortnightly intervals. When not in use diets were stored in sealed plastic containers at room temperature.

Experimental design and conduct

Groups of 15 rats of each sex were given diets containing 0 (control), 0·2, 1 or 5% (w/w) gum karaya for 13 wk. The animals were observed daily for abnormalities of condition or behaviour and were weighed on the first day of treatment, at weekly intervals thereafter up to wk 13 and again on the day of autopsy. The food consumption of each cage of animals was measured weekly throughout the study. Water intake was measured daily during wk 1–4 and 9–12 of the study.

The eyes of all animals were examined before treatment started using a Keeler direct ophthalmoscope. A mydriatic agent (Mydriacyl, 1%, Alcon Labs. Inc., USA) was instilled into the eyes 15 min before examination. This procedure was repeated during wk 13 for all control animals and for all animals given 5% gum karaya.

Blood samples were collected by orbital sinus puncture under light ether anaesthesia (diethyl ether, Analar grade, BDH, Poole, Dorset) following a 16-hr fast at wk 5 and 13 of treatment. On each occasion ten rats of each sex from each of the control and highest dietary level groups were sampled. Blood samples were examined for haemoglobin concentration, mean cell volume, total erythrocyte count, total and differential leucocyte count; packed cell volume, mean cell haemoglobin and mean cell haemoglobin concentration were calculated. Plasma was analysed for the concentration of blood urea nitrogen, glucose, total protein and albumin, sodium ions, potassium ions and for the activities of alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase.

Urine was collected from the same animals, during a 16-hr period of water deprivation, at wk 5 and 13 of treatment. Specimens were examined for microscopic constituents and for concentration of glucose, protein, reducing substances, ketones, bilirubin, urobilinogen and haemoglobin. The volume and specific gravity were also measured.

To minimize any bias introduced by these investigations a similar number of animals from the low and intermediate dietary level groups were deprived of food and water in the same manner but without the collection of biofluids.

At the end of the treatment period the animals were killed by ip injection of pentobarbitone sodium and were exsanguinated from the renal artery. An autopsy was performed, during which any macroscopic abnormalities were noted and the adrenal glands, brain, gonads, heart, kidneys, liver, lungs and thyroids were weighed. Samples of these organs and of aorta, caecum, colon, duodenum, epididymides, eyes, femur,

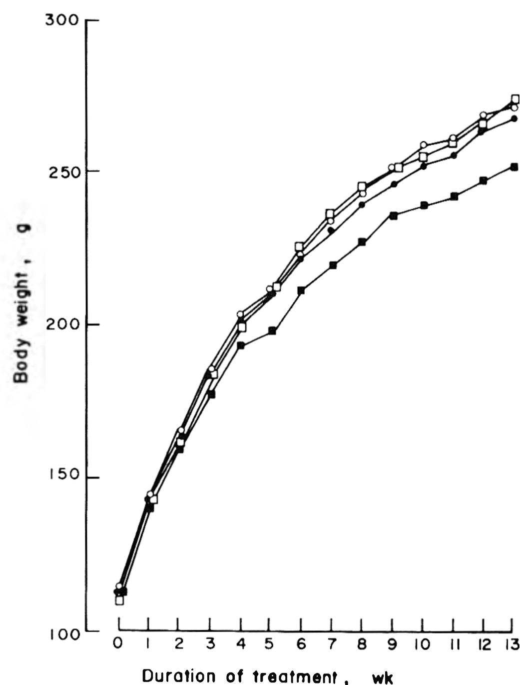

Fig. 2. Mean body weights of female rats fed diets containing 0(○), 0·2(●), 1(□) or 5(■)% gum karaya for 13 wk.

Table 1. *Mean food conversion efficiency of rats fed diets containing 0–5% gum karaya for 13 wk*

Dietary level (%)	Food conversion efficiency* (%) during wk . . .												
	1	2	3	4	5	6	7	8	9	10	11	12	13
							Males						
0	37·83	28·82	26·66	19·61	12·16	13·44	11·09	11·36	10·37	7·91	5·71	6·70	1·19
0·2	37·43	28·52	25·45	20·62	11·26	14·85	12·32	10·69	8·95	9·08	4·17	8·51	3·81
1·0	36·72	28·50	26·60	19·35	14·61	13·27	12·64	10·29	10·29	7·14	5·10	7·70	3·32
5·0	36·51	27·80	25·14	17·89	8·06	14·90	13·11	10·65	7·96	8·47	5·79	6·60	1·18
							Females						
0	26·65	15·58	14·90	12·53	6·23	8·52	7·45	5·97	5·48	5·17	1·78	4·73	2·55
0·2	24·49	16·65	14·73	12·81	6·47	8·03	6·38	5·32	4·69	5·00	2·00	5·22	2·99
1·0	25·87	16·12	15·28	12·16	7·73	9·45	6·74	5·69	4·53	3·38	2·86	4·56	5·97
5·0	23·38	15·26	13·74	11·64	3·74	8·94	6·14	5·51	6·16	2·10	1·85	4·53	3·63

*The percentage food-conversion efficiency is calculated as [weekly body-weight gain (g)/weekly food consumption (g)] × 100.

Table 2. *Mean water intake of rats fed diets containing 0–5% gum karaya for 13 wk*

Dietary level (%)	Water intake (ml/rat/day) during wk . . .							
	1	2	3	4	9	10	11	12
				Males				
0	26·6	28·5	31·1	35·2	31·9	32·6	33·6	32·5
0·2	25·3	28·9	31·7	36·5	35·3	34·5	36·3	36·0
1·0	30·2	31·9	35·2	34·2	34·5	34·4	36·3	35·3
5·0	33·1*	35·1*	35·8	34·6	35·4	34·9	37·5	36·5
				Females				
0	21·5	23·5	26·7	29·2	33·8	35·0	36·9	38·4
0·2	20·0	23·0	25·3	26·2	29·5	28·4	31·6	32·0
1·0	21·0	24·0	26·6	28·4	33·6	35·4	35·5	36·3
5·0	25·2*	26·6	27·7	28·9	34·6	35·3	35·6	34·5

Values are means for groups of 15 animals; those marked with asterisks differ significantly (Student's *t* test) from the corresponding control value (* $P < 0·05$).

heart, ileum, jejunum, lymph nodes, oesophagus, pancreas, pituitary, prostate, salivary gland, sciatic nerve, seminal vesicle, skin, skeletal muscle, spleen, spinal cord, stomach, thymus, tongue, trachea, urinary bladder and uterus were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues from control animals and those given 5% gum karaya were stained with haematoxylin and eosin for microscopic examination. Further sections of kidney from these animals were stained with Oil Red O and treated with Per-

Table 3. *Group mean haematological data of rats fed diets containing 0 or 5% gum karaya for 13 wk*

Sex and dietary level (%)	Hb (g/100 ml)	RBC (10^6/mm^3)	PCV (%)	MCH (pg)	MCHC (%)	MCV (cμm)	Leucocytes* (10^3/mm^3)				
							Total	N	L	M	E
					Wk 5						
Male 0	16·6	7·30	46	22·7	36	63	15·80	0·93	14·81	0·04	0·01
5	16·4	7·47	46	22·0	36	61	14·12	1·61	12·47	0·04	0·00
Female 0	16·4	7·32	46	22·5	36	62	13·07	1·21	11·82	0·01	0·03
5	16·6	7·24	45	22·9	37	62	13·84	1·24	12·60	0·00	0·00
					Wk 13						
Male 0	16·6	8·33	41	19·9	40	50	13·83	1·32	12·42	0·04	0·05
5	16·3	8·23	41	19·7	40	50	16·40	2·48	13·80	0·03	0·08
Female 0	15·8	7·77	40	20·4	39	52	11·76	1·37	10·32	0·02	0·03
5	15·8	7·73	40	20·5	40	52	12·28	0·79	11·44	0·02	0·03

Hb = Haemoglobin RBC = Red blood cells PCV = Packed cell volume MCH = Mean corpuscular haemoglobin MCHC = Mean corpuscular haemoglobin concentration MCV = Mean corpuscular volume N = Neutrophils L = Lymphocytes M = Monocytes E = Eosinophils

*No basophils were detected.

iodic acid Schiff reagent. Histopathological examination was restricted to these two groups, tissues from animals given 0·2 or 1% gum karaya being retained in fixative.

RESULTS

The only treatment-related clinical change was a tendency for treated animals, particularly those given 5% gum karaya, to show an increase in faecal bulk. This was noted from the onset of treatment and persisted for 13 wk.

During wk 4 and 5 the rate of body-weight gain in both sexes of rats given 5% gum karaya was reduced when compared with that of the controls. Subsequently, the males gained weight at a similar rate to the controls but the females given 5% karaya continued to show slight growth retardation. By the end of the study the mean weight of the males and females at this dietary level was lower than the controls by 5% and 12% respectively (Figs 1 and 2). The difference in weight gain over the 13 wk period between females given 5% gum karaya and the controls was statistically significant (Student's t test, $P < 0.05$). There was no adverse effect on food intake and the overall food conversion efficiency of animals given 5% karaya was only marginally lower than that of the controls (Table 1). With the exception of wk 4, male rats given 1 or 5% gum karaya drank more than the controls during all periods of measurement. The most marked increases were those shown during wk 1 and 2 by males given 5% gum karaya, when the differences from controls were statistically significant. The only significantly increased value for females was that during wk 1 for rats given 5% gum karaya (Table 2). Mean intakes of the test material over the entire test period, as calculated from body weight and food intake data, were 157, 791 and 3947 mg/kg/day for males and 189, 944 and 4754 mg/kg/day for females fed dietary levels of 0·2, 1 and 5% respectively.

Ophthalmoscopic examination revealed no ocular abnormalities that could be ascribed to administration of the test material. No haematological changes, disturbances of plasma analyses or effects on urine composition were found at either the wk 5 or 13 investigations. (Tables 3 and 4). The mean relative brain weight of females given 5% gum karaya was statistically significantly increased when compared with the control value ($P < 0.01$). No other organ weight changes were observed (Table 5).

The histopathological examination revealed peribronchial and perivascular lymphoid reactions associated with focal alveolitis in the lungs and intralobular lymphoid foci in the liver. Less common findings included renal cortical tubule regeneration, lymph-node hyperplasia, thyroid ultrimobranchial rests and foam-cell accumulation in the lungs. However, the distribution of these findings was similar in treated and control rats.

DISCUSSION

The tendency for treated animals to show increased faecal bulk and increased water intake might be related to the hygroscopic nature of gum karaya, but

Table 4. Group mean clinical chemistry data of rats fed diets containing 0 or 5% gum karaya for 13 wk

Sex and dietary level (%)	GOT (U/litre)	GPT (U/litre)	AlkPase (U/litre)	Na^+ (mequiv./litre)	K^+ (mequiv./litre)	Glucose (mg/100 ml)	BUN (mg/100 ml)	Protein: Total (g/100 ml)	Protein: Albumin (g/100 ml)	A:G ratio
Wk 5										
Male 0	118	32	556	143	3·6	88	13	6·5	3·7	1·3
5	183	57	521	143	3·3	96	13	6·5	3·5	1·2
Female 0	92	28	331	141	3·3	101	14	6·8	3·9	1·4
5	98	26	351	141	3·2	108	15	6·6	3·8	1·4
Wk 15										
Male 0	98	41	253	141	3·5	129	13	6·5	3·5	1·2
5	89	34	237	141	3·6	137	14	6·5	3·4	1·1
Female 0	87	45	138	140	3·4	123	15	7·4	4·2	1·3
5	71	33	153	140	3·3	135	15	7·1	3·9	1·2

GOT = Glutamic-oxalacetic transaminase GPT = Glutamic-pyruvic transaminase
AlkPase = Alkaline phosphatase BUN = Blood urea nitrogen
A:G = Albumin:globulin

Table 5. *Group mean organ weight data of rats fed diets containing 0 or 5% gum karaya for 13 wk*

Sex and dietary level (%)	Body weight (g)	Relative organ weights (% of body weights)							
		Brain	Liver	Heart	Gonads	Adrenals	Thyroids	Kidneys	Lungs
Male 0	488	0·412	3·197	0·305	0·723	0·012	0·007	0·569	0·366
5	470	0·427	3·077	0·297	0·759	0·012	0·007	0·567	0·348
Female 0	266	0·684	3·367	0·353	0·026	0·024	0·010	0·643	0·451
5	248	0·744*	3·238	0·359	0·029	0·026	0·010	0·630	0·478

Values are means for groups of 15 animals; that marked with an asterisk differs significantly (Student's t test) from the corresponding control value (*$P < 0·05$).

in the absence of any information on faecal weight and moisture content, this can only be speculated.

In the absence of any reduction in food consumption or other manifestations of toxicity, the slight retardation of weight gain shown by animals given 5% gum karaya was probably related to a nutritional imbalance, caused by the incorporation of a relatively high level of test article. At lower dietary levels of karaya animals of both sexes showed comparable or slightly greater weight-gains than control animals. The increase in relative brain weight seen in females given 5% gum karaya is considered to have resulted from this slight growth retardation since other workers have shown that brain weight is primarily a function of age and alters little with changes in body weight (Feron, de Groot, Spanjers & Til, 1973; Oishi, Oishi & Hiraga, 1979; Scharer, 1977).

In summary, since no adverse effects were noted on food intake or clinical condition and since no adverse effects were revealed by haematological, blood chemistry and urine analyses, ophthalmoscopy, organ weight analysis or histopathology, a dietary level of 5% gum karaya can be regarded as a no-untoward-effect level. Since this level corresponded to an average intake of about 4 g/kg/day it is suggested that 40 mg/kg/day could be regarded as an acceptable daily intake (ADI).

Current usage of gum karaya in the UK food industry is estimated by INGAR at 100 tons/year. Assuming a population of 55 million, this gives an average intake of 1·82 g/person/year, or about 5 mg/day. For a 70-kg man this represents an intake some 560 times lower than the ADI proposed above. It would appear therefore, that no toxicological hazard is posed by the present usage of gum karaya in foodstuffs.

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SHORT-TERM TOXICITY OF 2-PHENYLPROPAN-1-OL (HYDRATROPIC ALCOHOL) IN RATS

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Abstract—Phenylpropan-1-ol was added to the diet of groups of 15 male and 15 female rats to provide intakes of 0 (control), 10, 40 or 160 mg/kg/day for 13 wk. No effects on body weight, food intake, water intake, haematology, semi-quantitative analysis of urine, renal concentration and dilution tests, serum chemistry or the histological examination could be attributed to treatment. Increased liver weights at the highest dose level in both sexes and increased kidney weights at the two highest dose levels in males were considered to be related to treatment, but in view of the lack of histological abnormalities this study did not demonstrate whether these were toxic or adaptive effects. An isolated renal mesenchymal tumour was found in the females fed the highest dose level. It is concluded that the no-effect level in this study was 10 mg/kg/day.

INTRODUCTION

2-Phenylpropan-1-ol (hydratropic alcohol; FEMA no. 2732; CoE no. 2257) is used as a component of fragrances and food flavourings. Opdyke (1975) estimated that its use in fragrances in the USA was less than 500 kg/yr and that the normal percentage concentrations (maximum concentrations in brackets) were 0·01 (0·15) in soap, 0·001 (0·015) in detergents, 0·01 (0·05) in creams and lotions and 0·1 (0·6) in perfume. The use in food, worldwide, has been calculated as 25 kg/yr, with normal concentrations (ppm) of 7·2 (8·9) in non-alcoholic beverages, 5·7 (6·8) in ice cream, 7·3 (10·0) in candy, 13·4 (15·5) in baked goods, 10·6 (12·0) in gelatin desserts and 1·2 (1·6) in chewing gum (Grundschober, Hall, Stofberg & Vodoz, 1975), the figures in brackets again representing the highest concentrations reported.

The small amount of published information on the toxicity of 2-phenylpropan-1-ol was summarized by Opdyke (1975). The data quoted show that metabolism largely involves oxidation to the acid followed by excretion as the glucuronide conjugate, although a proportion (10–20%) is excreted as the direct glucuronide conjugate. The high proportion excreted as the acid conjugate is in keeping with the established pathway of metabolism of hydratropic acid (Dixon, Caldwell & Smith, 1977). These workers showed also that this pattern of metabolism of the acid applied in man as well as the rat. The LD_{50} values quoted by Opdyke (1975) are 2·3 g/kg in rats treated orally and in excess of 5 g/kg in rabbits treated by the dermal route; the material has been found to be non-irritant at full strength in rabbits and at a concentration of 6% in petrolatum in humans. The latter formulation also proved to be without sensitizing potential in humans.

The objective of the present study was to obtain subacute data appropriate to the safety evaluation of 2-phenylpropan-1-ol, particularly with reference to its use as a food additive.

EXPERIMENTAL

Test material. 2-Phenylpropan-1-ol was supplied by the International Organization of the Flavor Industry, Geneva. The material complied with the following specification: viscous liquid; relative density (d_{20}^{20}), 1·000–1·006; refractive index (n_D^{20}), 1·5240–1·5280; acid value, max 1; assay, min 95%. Analysis of the sample used gave the following results: relative density (d_4^{20}), 1·0031; refractive index (n_D^{20}), 1·5251; assay (GLC), 99·2%. The sample was stored in closed containers at 4°C.

Animals and diets. Weanling rats were obtained from a specified-pathogen-free colony of the Wistar strain (Olac 1976 Ltd, Bicester, Oxon). They were housed in groups of five of the same sex and treatment, in plastics and stainless-steel grid-floored cages (North Kent Plastic Cages Ltd, Dartford, Kent) suspended on racks with paper for collection of excreta. The cages were kept in an air-conditioned room and daily measurement of maximum and minimum temperatures showed 91% of the values to be in the 24 ± 3°C range. All of the daily relative humidity measurements were in the 55 ± 10% range. The weanling rats were acclimatized to the environmental conditions for 9 days before receiving the test diets.

The rats were divided into four groups of 15 males and 15 females and the dietary concentrations of the test material were adjusted, on the basis of food-intake and body-weight measurements, to provide as nearly as possible a constant intake of 0 (control), 10, 40 and 160 mg 2-phenylpropan-1-ol/kg body weight/day for the four groups throughout the 13-wk feeding period. The basic diet was Laboratory Animal Diet No. 2 (Spratt's Patent Ltd, Barking, Essex); fresh test diets were prepared at weekly intervals and stored in closed metal containers.

Test procedure. The rats were observed daily for abnormalities of condition. They were weighed initially and then twice weekly throughout the study.

Food and water intakes were recorded for each cage of rats for 3 days before treatment and at twice-weekly intervals during the experiment.

Blood, collected from the retro-orbital sinus at wk 6 and from the aorta during the anaesthetic phase prior to the post-mortem examination, was examined for haemoglobin concentration, erythrocyte count, packed cell volume and leucocyte count. Counts of reticulocytes and the different types of leucocytes were confined to the samples from the control and highest dose groups. Serum was separated from the blood collected at the end of the study and was analysed for glucose, urea, protein, albumin, glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

Urine was collected over a 6-hr period, from each rat during wk 6 and during the last week of the experiment. It was examined for volume, specific gravity and pH, and semi-quantitative analyses for glucose, blood, bile, ketones and protein were carried out. Renal concentrating and diluting ability was examined further by measuring the specific gravity and volume of the urine collected during a 2-hr period immediately after a 25-ml/kg oral water load and of that collected during a 4-hr period starting 16 hr after the water load. The number of cells was counted in the samples collected immediately after the water load.

After the feeding period (on days 95–97 for males and days 98, 99 and 102 for females), the animals were fasted overnight, killed by exsanguination under barbiturate anaesthesia and subjected to a post-mortem examination. Any abnormalities were noted and the adrenal glands, brain, caecum (with and without contents), gonads, heart, kidneys, liver, pituitary, spleen, stomach and thyroid were weighed. Samples of these tissues and of urinary bladder, colon, diaphragm, epididymis, eye, Harderian gland, lung, lymph nodes, mammary gland, nerve, oesophagus, pancreas, prostate, rectum, salivary gland, seminal vesicles, skeletal muscle, skin, small intestine, spinal cord, trachea, thymus, uterus and vagina were preserved. Those from the highest dose level and control groups were sectioned and examined microscopically.

RESULTS

All of the animals survived the treatment period and no abnormalities of condition or behaviour were seen. The group mean body weights (Table 1) showed no consistent or dose-related reductions in the males. Statistically significant deviations from the control (not shown in Table 1) occurred during the first 2 wk in the lowest dose group and during days 67–84 in the intermediate dose group. In females, the mean body weights were lower in all the treated groups than in the control group from wk 3, but the differences were not dose related and did not increase markedly with time. For example, the value for the group given 40 mg/kg was 5% lower than the control value at day 21 and 8% lower by day 91.

The mean food intakes of the male treated groups over the period of the study (Table 1) were slightly lower than those of the controls but not in a dose-related manner, the only statistically significant differ-

Table 1. *Summary of dietary concentrations, body weights and food, water and flavouring intakes for rats fed diets to provide intakes of 0–160 mg 2-phenylpropan-1-ol/kg/day for 13 wk*

Nominal dose level (mg/kg/day)	Body weight (g) at wk 0†	2	4	6	8	10	13	Mean food intake‡ (g/rat/day)	Mean water intake‡ (ml/rat/day)	Flavouring consumption: Dietary concn§	Flavouring consumption: Calculated intake‖
							Males				
0	102	199	286	326	374	409	438	23.3	33.8	—	
10	97	196	283	328	371	406	439	22.7	31.5*	70–200	9.7 (7.9–10.9)
40	101	193	275	316	354	388	420	21.7**	33.3	280–800	38.2 (29.8–41.8)
160	98	197	278	322	370	404	447	22.6	32.4	1120–3200	155 (122–175)
							Females				
0	94	154	198	224	244	257	275	18.5	28.9	—	
10	91	152	188**	213**	231**	241**	256**	18.6	36.9***	70–140	10.1 (9.2–11.3)
40	90	147*	185**	210**	228**	238**	254**	17.9	30.5	280–560	39.7 (36.0–47.3)
160	90	151	188*	213*	227**	238**	251**	16.9***	34.4***	1120–2600	156 (132–190)

†Wk 0 = first day of treatment.
‡Means of twice-weekly measurements.
§Range of concentrations used during the study.
‖Means (and ranges) of twice-weekly calculations from mean body weight, food intake and dietary concentration.
Values marked with asterisks differ significantly (t test) from the control: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

ence being at the intermediate dose. The twice-weekly measurements for this group were generally 5–10% lower than those for the control group, the differences often reaching statistical significance. Most of the food-intake values for the other male groups were within 5% of the control and only scattered statistically significant differences were found. In the females the food intake of the lowest dose level was similar to that of the controls and in the 40 mg/kg/day group there were only small, non-significant differences in both the twice-weekly and overall values. The food intake of the highest dose group was lower from wk 4, resulting in a lower overall mean value.

The range of dietary concentrations used and the resulting average intakes of phenylpropanol-1-ol are shown in Table 1. The values calculated at twice-weekly intervals showed that 96, 92 and 96% of those for the 10-, 40- and 160-mg/kg/day males were within 10% of the desired dosage. For females, the corresponding figures were 88, 88 and 65%.

The overall water intakes for the males given the two higher dose levels were similar to the control intake, but at the 10-mg/kg/day dose the overall intake was lower. This reflected the values throughout the study, including the pretreatment period, although generally the differences during the experiment were not statistically significant. The pattern of water intake by the females was different, with higher intakes by all treated groups although there was no dose relationship.

There were inconsistent differences in the results of the haematological examinations at wk 6 and 13 (Table 2). The erythrocyte counts were lower than the control counts in the high-dose males at the end of the study, whereas in the corresponding females at wk 6 this same measurement and the haemoglobin concentration were significantly greater than the control values. The data for the intermediate dose levels are not shown in Table 2, but the only statistically significant deviation from the control was a higher erythrocyte count 7·97 × 10^6/mm^3 in the intermediate-dose males at the first examination.

No urine samples gave reactions for bilirubin or glucose at either wk 6 or wk 13, and only occasional samples gave positive reactions for blood. The incidence of these did not differ significantly between the treated and control groups. There were some differences in the incidences of samples with various reactions for protein (Table 3). Significantly more samples from the males given 40 mg/kg/day for 6 wk showed an intermediate (+ +) reaction with an associated decrease in the number of samples giving the higher-grade reactions. In the females there were more samples with the minimal grade of reaction from the lowest dose group at wk 13 and from the 160-mg/kg/day group at wk 6. Further statistical analysis of the latter group revealed that the total incidence of animals giving the higher grades of reaction for protein (+ + or greater) was significantly less than the control incidence.

The results of the renal concentration and dilution tests in the control and highest dose group (Table 4) were similar, as were the results for the lower dose groups and control. The only statistically significant differences were slightly lower mean volumes of the samples collected at 0–2 hr (5·0 ml) and 16–20 hr (0·4 ml) from the 40-mg/kg/day females at wk 13 and a low specific gravity (1·057) for the samples taken

Table 2. *Results of haematological examinations of rats fed diets to provide intakes of 0 or 160 mg 2-phenylpropan-1-ol/kg/day for 6 or 13 wk*

Dose level (mg/kg/day)	RBC (10^6/mm^3)	Hb (g/100 ml)	PCV (%)	Retics (% RBC)	Leucocytes: Total (10^3/mm^3)	Differential: N	E	L	M
				Wk 6					
Male									
0	7·36	17·3	49†	0·8	30·2	13·7	0·6	85·2	0·7
160	6·96	17·3	47	0·7	28·4	15·1	0·8	83·2	1·0*
Female									
0	7·48	17·5	48	1·1	18·4	9·5	1·0	89·3	0·3
160	8·15*	18·4*	47	1·2	14·8	11·4	0·7	87·0	1·0
				Wk 13					
Male									
0	8·04	14·8	49	1·4	4·6	23·1	1·3	74·8	0·8
160	7·47*	14·6	49	1·5	4·2	18·8	0·9	79·5	0·7
Female									
0	7·34	14·4	45	1·6	3·7	17·8	0·8	79·8	0·8
160	7·33	14·3	45	1·4	3·5	19·9	1·9	76·9	1·3

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

†Mean for 12 rats only.

The figures are means for groups of 15 rats except where stated otherwise. Those marked with an asterisk differ significantly (0·05 > P > 0·01 by t test) from the corresponding controls.

Basophilic leucocytes were not encountered in the differential counts.

Table 3. *Incidence of various reactions for protein in urine samples from rats fed diets to provide intakes of 0-160 mg 2-phenylpropan-1-ol/kg/day for 6 or 13 wk*

Dose level (mg/kg/day)	Grade of reaction†...	Males t	Males +	Males ++	Males +++	Males ++++	Females t	Females +	Females ++	Females +++	Females ++++
					Wk 6						
0		1	7	4	3	0	1	8	5	1	0
10		1	5	6	3	0	4‡	8	3	0	0
40		0	5	10*	0	0	3	7	5	0	0
160		2	5	8	0	0	6*	8	1	0	0 (a)
					Wk 13						
		0	6	8	0	1	1	7	6	1	0
10		0	3	10	2	0	6*	6	3	0	0
40		1	2	10	1	1	3	9	3	0	0
160		0	7	6	2	0	1	7	7	0	0

†Reactions on test strips defined as: t, trace; +, c. 30 mg/100 ml; ++, c. 100 mg/100 ml; +++, c. 300 mg/100 ml; ++++, c. 1000 mg/100 ml.
‡Includes one sample with no reaction for protein.
Figures marked with an asterisk differ significantly (0·05 > P > 0·01 by Fisher's exact test) from the control; (a) indicates that the total with grade ++ or above was significantly less than in the control group.

from the low-dose females at 0–6 hr, again at wk 13. The mean pH values for the urines of both sexes given 160 mg phenylpropanol/kg/day were significantly lower than the control values at wk 6, but the difference was much less marked after 13 wk (Table 4). There was no similar finding at the intermediate dose level (pH 7·2 and 6·4 in males and females, respectively, at wk 6). The urinary cell count was slightly higher in the top-dose female group than in the controls at wk 6 and markedly higher at wk 13. However, the latter count was due entirely to one animal with a value of 27·5 × 10^3/hr, and when this was excluded the group mean was 0·5 × 10^3 cells/hr, close to that for the controls.

There were no statistically valid differences between the control and high-dose rats in the results of the serum analyses. With the lower doses, the only statistically significant differences were slightly higher

Table 4. *Results of renal concentration and dilution tests, urinary pH determinations and urinary cell counts in rats fed diets to provide intakes of 0 or 160 mg 2-phenylpropan-1-ol/kg/day for 6 or 13 wk*

Dose level (mg/kg/day)	0–2 hr† Specific gravity	0–2 hr† Volume (ml)	0–2 hr† Cells (10^3/hr)	0–6 hr Specific gravity	0–6 hr Volume (ml)	0–6 hr pH	16–20 hr† Specific gravity	16–20 hr† Volume (ml)
				Wk 6				
Male								
0	1·010	6·9	1·0	1·042	2·9	7·1	1·083	0·7
160	1·009	7·3	1·1	1·046	3·6	6·5*	1·085	0·7
Female								
0	1·007	4·7	0·5	1·063‡	1·5	6·8	1·081	0·4
160	1·008	4·9	1·1*	1·050	1·9	6·2**	1·077	0·4
				Wk 13				
Male								
0	1·010	10·5	0·7	1·067‡	2·5‡	7·3	1·074	0·7
160	1·011	9·1	0·5	1·068	2·8	6·9	1·068	0·8
Female								
0	1·010	6·1	0·5	1·075	1·7	6·4	1·126‡	0·5
160	1·011	5·5	2·4	1·069	2·1	6·2	1·110	0·7

†Samples collected over the stated period following intake of a 25-ml/kg oral water load at 0 hr.
‡Mean of 14 measurements.
The figures are means for groups of 15 rats except where indicated otherwise. Those marked with asterisks differ significantly (t test) from the control value: * = P < 0·05; ** = 0·01 > P > 0·001.

Table 5. *Results of analysis of serum from rats fed diets to provide intakes of 0–160 mg 2-phenylpropan-1-ol/kg/day for 13 wk*

Dose level (mg/kg/day)	GOT (IU)	GPT (IU)	LDH (IU)	Urea (mM)	Protein (g/100 ml)	Albumin (g/100 ml)	Glucose (mM)
				Males			
0	67·0	18·6	1563	8·3	6·5	3·7	6·6
10	66·6	20·7	1645	8·3	7·0*	3·7	6·3
40	69·3	18·0	1761	8·0	6·7*	3·7	5·8
160	66·1	28·4	1354	7·9	6·6	3·8	7·2
				Females			
0	55·6	14·9†	1204	9·5	7·0	4·1	4·4
10	60·2†	16·1	1224	8·4	6·7	3·8*	4·3
40	62·8	15·7	1226	9·2	6·9	4·1	4·4
160	59·3	20·3	1112	9·5	7·1	4·2	4·6

GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase LDH = Lactic dehydrogenase IU = International units

†Mean of 14 determinations.

The figures are means for groups of 15 animals except where indicated otherwise. Those marked with an asterisk differ significantly (t test) from the control value: $0.05 > P > 0.01$.

protein concentrations in the males of both groups and a lower concentration of albumin in the low-dose females (Table 5).

The relative liver weights of both sexes given 160 mg/kg/day were higher than those of the controls (Table 6). The actual weights were also higher than the controls but the difference was statistically significant only in the males. The same group of males had absolute and relative kidney weights approximately 15% higher than the control values, whilst at the intermediate dose level (40 mg/kg/day) the relative kidney weight was approximately 7% higher than the control with no significant difference in the absolute weight. The absolute and relative kidney weights of the high-dose females were 13–15% higher than those of the control group. These differences were not statistically significant and were due to very high values from one animal. Exclusion of these weights resulted in mean values within 5% of the control.

The males on the intermediate dose level showed higher relative heart and pituitary weights with no significant increase in the absolute weights. These values showed statistical significance by the t test but there was no overall significance using the analysis of variance involving all the groups. Comparison with the controls showed a higher mean relative caecal weight in the high-dose females, a high relative adrenal weight in the low-dose females, and a high relative brain weight in all the female groups (but showing no dose relationship). None of these findings were associated with a significant difference in the absolute weights.

The range and severity of the histological findings were similar in the control and high-dose animals and were those to be expected in these animals. Increases in the incidence of lesions were confined to the lung; there were more treated females with peribronchial mononuclear cell cuffing (eight affected compared with two controls) and in the males the incidence of moderate alveolar thickening was higher in the treated group (in ten rats compared with three controls), although the total incidence of treated animals with any grade of alveolar thickening was not significantly increased over that of the controls (15 *v.* 10). The female high-dose animal with a high kidney weight proved to have a mesenchymal tumour in the kidney. Other findings in the renal system of this rat were severe nephrosis, chronic inflammatory-cell infiltration and pelvic epithelial hyperplasia, together with hyperplasia of the urinary bladder.

This animal had also shown other signs of renal abnormality in the form of a high serum-urea concentration (22 mM compared with a control mean of 9·5 mM), low specific gravity and high urine volumes in the concentration tests and a high urinary pH and cell count (8·0 and 27·5, respectively, compared with control means of 6·4 and 0·5). It also showed a strong reaction for blood in the urine at wk 13.

DISCUSSION

The lower body weight of all the groups of treated females is not considered to be a toxic effect. There was no parallel effect in the males and neither the time of onset nor the magnitude of the differences from the control were dose related. The group mean weight of the three treated groups did not differ by more than 5 g at any time and the highest dose level was the last to be affected. In addition the differences from the control, although statistically significant, were small (7–9% of the control). Such a pattern of small differences with no dose relationship and confined to one sex is unlikely to represent an effect of treatment. It is more likely that the control group showed an anomalously high weight gain.

The differences in weight gain cannot be correlated with the food intake, which was lower in the females on the highest dose but not markedly low in the females on the intermediate and low doses despite the similarity of the body weights in these three groups. A reason for the variations in food intake may have been that the diets were somewhat unpalatable. However, there was little effect in the high-dose males, which consumed the highest dietary concentrations, and there were no differences at the beginning of the study when such effects are usually most obvious.

Table 6. *Organ weights expressed relative to body weight of rats fed diets to provide intakes of 0–160 mg 2-phenylpropan-1-ol/kg/day for 13 wk*

Dose level (mg/kg/day)	Terminal body weight (g)	Relative organ weight (g/100 g body weight)						Caecum					
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Full	Empty	Gonads†	Pituitary‡	Adrenals‡	Thyroid†
Males													
0	418	0·49	0·25	2·42	0·18	0·54	0·36	0·75	0·25	0·82	2·3‡	10·6	3·4
10	418	0·50	0·26	2·46	0·18	0·55	0·36	0·81	0·26	0·80	2·4	10·2	3·7
40	397	0·51	0·27*	2·41	0·18	0·58**	0·37	0·76	0·27	0·84	2·6*	10·1	3·6
160	418	0·50	0·26	2·83**	0·17	0·63***	0·37	0·76	0·26	0·79	2·4	10·6	3·9
Females													
0	257	0·76	0·29	2·36	0·22	0·58	0·44	1·19	0·32	49	5·0	19·7	5·1
10	240*	0·82**§	0·30	2·43	0·23	0·60	0·46	1·12	0·31	54	5·6	22·8*§	5·5
40	239*	0·82*	0·29	2·36	0·23	0·57	0·45	1·13§	0·30§	47	4·8	21·1	5·2
100	235**	0·83**	0·29	2·70***	0·24	0·67	048*	1·47**§	0·36*§	51	5·2	22·6	5·8

†Values for females expressed in mg/100 g body weight.
‡Values expressed in mg/100 g body weight.
§Mean for group of 14 animals.
Values are means for groups of 15 rats except where indicated otherwise. Those marked with asterisks differ significantly (Student's t test) from the control: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Although the erythrocyte counts of the males given 160 mg/kg/day were 5–10% lower than the control count, the other measurements on these cells provided no supporting evidence of an anaemia and the reticulocyte counts showed no evidence of any compensatory increase in red-cell turnover. In the absence of any similar findings in the females it is difficult to establish with certainty whether there was any effect on the erythrocytes.

There was no evidence of any functional defect in the kidneys and the slightly lower pH values may have been related to the excretion of the alcohol or its metabolites in the urine, since Williams (1959) reported this as the main route of excretion. The increases in renal cell excretion were not related to treatment since the high values were associated with one animal subsequently shown to have a renal tumour and hyperplasia of the bladder and renal pelvis. Despite the normal results in the tests of renal function, male animals given doses of 40 or 160 mg/kg/day had higher relative kidney weights without any detectable underlying pathology. It is possible that these weights reflected the increased demand to excrete the products of metabolism, but it is difficult to equate this with the sex difference since there was no increased renal weight in females after elimination of the one animal with grossly abnormal pathology.

An increased metabolic demand may have accounted for the higher liver weights at the highest dose level, since Williams (1959) showed that 2-phenylpropan-1-ol is either oxidized before glucuronide conjugation and excretion or conjugated directly to a hydroxyl glucuronide. If this explanation is correct it is unlikely to be associated with the conjugation stage of the metabolism since there was no increase in liver weight in rats given seven daily ip injections of 80 mg 2-phenylpropan-1-ol/kg despite a doubling of the glucuronide excretion (Lake, Longland, Harris *et al.* 1980).

An alternative explanation is that the increased liver and kidney weights were the result of a toxic effect, and since there are insufficient data from the present study to differentiate between these alternatives the higher organ weights must be attributed to treatment with 2-phenylpropan-1-ol.

The higher relative brain weights in females with no differences in the absolute weights and no corresponding finding in the males is likely to be a result of expressing the normal organ weight in relation to a low body weight. This effect in animals that have failed to grow as well as the controls is well documented (Feron, de Groot, Spanjers & Til, 1973; Oishi, Oishi & Hiraga, 1979). It is likely that a similar explanation applies to the slightly higher relative caecum weights.

It was unexpected to find a renal tumour in an animal of this age, especially as spontaneous tumours of the kidney are considered to be rare (Snell, 1967). Nevertheless they do occur; a review of 675 rats of each sex used as controls in 3-month studies in these laboratories revealed one kidney tumour (Gaunt, Farmer, Grasso & Gangolli, 1967). Despite this low background incidence, spontaneous mesenchymal tumours of the kidney are predominantly a neoplasm of the young rat, most having been reported in animals under 12 months of age (Hard, 1976). Although the tumour found in the present study was in a rat given 160 mg 2-phenylpropan-1-ol/kg, the absence of signs of renal damage in the other rats suggests that this lesion was not related to treatment.

This experiment has revealed few effects that can be definitely attributed to treatment. The liver- and kidney-weight increases are considered to be treatment-related, although it is uncertain whether these were toxic or adaptive effects. On this basis the no-effect level for this study was 10 mg/kg/day.

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MUTAGENICITY SCREENING OF POPULAR THAI SPICES

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Abstract—Mutagenicity screening was carried out on 31 samples of popular Thai spices derived from 12 different families of plants, namely the Amaryllidaceae (2), Graminae (1), Labiatae (4), Lauraceae (1), Magnoliaceae (1), Myristicaceae (2), Myrtaceae (2), Piperaceae (3), Rutaceae (2), Solanaceae (2), Umbelliferae (2) and Zingiberaceae (9). Two variations of the rapid streak method of *rec*-assay in *Bacillus subtilis* strains H17 (rec^+) and M45 (rec^-) were used. Only Ceylon cinnamon (the bark of *Cinnamomum zeylanicum* Nees of the family Lauraceae) showed mutagenic activity. The crude form of this spice and its water-heated and water-macerated residues all produced the *rec* effect, while water-heated and water-macerated filtrates did not, even in concentrations equivalent to as much as 50 mg solids/test disc.

INTRODUCTION

Spices have been used for centuries to preserve foods or to make them more appetizing by improving taste and/or aroma. Many spices have also been used in both folk medicines and modern drug preparations, since they contain active medicinal ingredients (Cantoria, 1976; Morton, 1980; Swinyard & Harvey, 1975). Because of these widespread uses, the chemical constituents, the pharmacological actions and the antimicrobial activities of spices have been widely studied (Dasgupta & Datta, 1980; Isogai, Suzuki & Tamura, 1976; Monsereenusorn, 1980; Prasad & Joshi, 1949). However, little work has been carried out on the possible carcinogenic properties of spices and this may be useful since many are consumed in fairly large quantities over long periods of time.

Mutagenicity is the capacity of a substance to induce changes in genes or chromosomes (Hollstein, McCann, Angelosante & Nichols, 1979) and many studies have revealed a close relationship between mutagenicity and carcinogenicity (Ames, Durston, Yamasaki & Lee, 1973; McCann, Choi, Yamasaki & Ames, 1975; Slater, Anderson & Rosenkranz, 1971). About 80–90% of the substances recognized as mutagenic also show carcinogenic action. Consequently, any spice showing a mutagenic effect may also have an associated carcinogenic capacity. In this investigation, 31 samples of popular Thai spices were screened for mutagenicity using the rapid streak method of the *rec*-assay system.

EXPERIMENTAL

Test spices. Thirty-one samples of fresh and dried Thai spices were bought from local markets and herb shops, respectively, in Bangkok. The spices and parts of the plants used are identified in Table 1. To reduce the residues of any insecticides that might be present, the fresh spices were washed twice, drained and finally ground. The dried spices were powdered directly, except for turmeric which was purchased in the powdered form. Each ground spice was divided into three portions for treatment according to the following three protocols, which reflected some of the cooking procedures to which the spices are commonly subjected and which together could provide some information on the heat stability and water solubility of active constituents of the spices:

(1) One portion was not treated and was designated the 'crude form' of the spice.
(2) The second portion was mixed with water (10 g spice in 50 ml, except turmeric for which 100 ml water was used) and the mixture was heated in a boiling water-bath for 1 hr, cooled to room temperature and filtered through gauze or cotton wool to yield the 'water-heated residue' and 'water-heated filtrate'. The latter was divided into two parts, one part remaining untreated while the other was dried at 85°C, 0·5–1 g of the residue being redissolved in 1 ml water.
(3) The third portion was treated in the same way as the second portion, except that the mixture was macerated for 5 days at room temperature instead of being boiled and the treated part of the 'water-macerated filtrate' separated from the 'water-macerated residue' was dried at 45°C rather than at 85°C.

Bacteria. Bacillus subtilis strain H17 (rec^+), possessing regular DNA recombination repair properties, and strain M45 (rec^-), a mutant with a much lower DNA-repair efficiency, were used as test organisms. These two strains were kindly provided by Dr T.

Table 1. *Tested spices and their plant sources*

Common name	Source	Family
	Fresh spices	
Bird pepper	Fruit of *Capsicum minimum* Roxb.	Solanaceae
Cherry pepper	Fruit of *Capsicum frutescens* var. *longum* Bail	Solanaceae
Galangal	Rhizome of *Alpinia galanga* Swartz	Zingiberaceae
Garlic	Bulb of *Allium sativum* Linn.	Amaryllidaceae
Ginger (old)	Rhizome of *Zingiber officinale* Roscoe	Zingiberaceae
(young)	Rhizome of *Zingiber officinale* Roscoe	Zingiberaceae
Hoary basil	Leaf of *Ocimum americana* Linn.	Labiatae
Holy basil	Leaf of *Ocium sanctum* Linn.	Labiatae
Kitchen mint	Leaf of *Mentha cordifolia* Apiz.	Labiatae
Kra chai	Root of *Gastrochilus panduratus* Ridl.	Zingiberaceae
Leech lime	Leaf of *Citrus hystrix* DC	Rutaceae
Lemon grass	Stem of *Cymbopogon citratus* Stapf.	Graminae
Shallot	Bulb of *Allium ascalonicum* Linn.	Amaryllidaceae
Sweet basil	Leaf of *Ocimum basilicum* Linn.	Labiatae
Zedoary	Rhizome of *Curcuma zedoaria* Rose	Zingiberaceae
	Dried spices	
Bay leaf	Leaf of *Pimenta racemoso* Moiller	Myrtaceae
Black pepper	Fruit of *Piper nigrum* Linn.	Piperaceae
Caraway	Fruit of *Cuminum cyminum* Linn.	Umbelliferae
Ceylon cinnamon	Bark of *Cinnamomum zeylanicum* Nees	Lauraceae
Chinese star anise	Fruit of *Illicium verum* Hook	Magnoliaceae
Clove	Flower of *Eugenia caryophyllus* Bullock et Harris	Myrtaceae
Coriander	Fruit of *Coriandrum sativum* Linn.	Umbelliferae
Dee plee	Fruit of *Piper chaba* Hunt	Piperaceae
Mace	Arillode of *Myristica fragans* Linn.	Myristicaceae
Nutmeg	Seed of *Myristica fragans* Linn.	Myristicaceae
Phlai	Rhizome of *Zingiber cassumunar* Roxb.	Zingiberaceae
Phrik hom	Fruit of *Zanthoxylum limonella* Alston	Rutaceae
Siam cardamom	Fruit of *Amomum krervanh* Pierre	Zingiberaceae
Turmeric	Rhizome of *Curcuma longa* Linn.	Zingiberaceae
White pepper	Fruit of *Piper nigrum* Linn.	Piperaceae
Wild ginger	Rhizome of *Zingiber zerumbet* Smith	Zingiberaceae

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Media and culture preparation. The composition of the liquid medium used for bacterial culture was 10 g beef extract (Difco Laboratories, Detroit, MI, USA), 10 g polypeptone (Daiko Chemical Co., Osaka, Japan) and 5 g NaCl (Sigma Chemical Co., St. Louis, MO, USA) in 1 litre distilled water, pH 7. For solid medium, 15 g bacto-agar (Difco Laboratories) was added and 10 ml medium was dispensed into each 10-cm Petri dish.

Culture preparations. B. subtilis strains H17 and M45 were separately cultivated in liquid medium at 37°C for 18 hr in a shaker incubator, and 50% (w/v) glycerol was then added to each culture (glycerol:culture = 1:3). The cultures were then divided into small vials and kept in the freezer at −80°C. One vial was used for each experiment.

Mutagenicity screening of spices. The rapid streak method of the *rec*-assay system described by Kada and his colleagues (Kada, Moriya & Shirasu, 1974; Kada, Tutikawa & Sadaie, 1972) was used with slight modification. Briefly, the frozen cultures of *B. subtilis* strains H17 and M45 were thawed at room temperature, each strain was streaked on the agar plate in a long line and spices were tested using 15-mm paper discs. These discs were wetted with 0·05 ml water before application of the sample. For testing of the crude samples and residues (water-heated and water-macerated), the wetted disc was placed on the agar, with the margin of the paper touching the two streaked lines, and a 15-mm diameter and 5-mm high metal ring was placed on top of the disc for easy loading with the spice powder or residue. After loading, the ring was removed. For the test filtrates (water-heated and water-macerated), a volume of 0·05 ml was pipetted/inoculated into the wetted disc before the disc was put on the agar. In this case the concentrated filtrate gave approximately 25–50 mg solids/disc.

Mitomycin C (Kyowa Hokko Kogko Co. Ltd, Tokyo, Japan) at 0·05 μg/disc was used as the positive control, while water and cellulose (Sigmacell® Type 100; Sigma Chemical Co.) were used as negative controls.

Two sets of plate tests were carried out and each set was duplicated. The first set was incubated at 37°C for 24 hr (the 'standard streak method'). The second set was first put in the refrigerator (4°C) overnight and then incubated continuously at 37°C for 24 hr (the 'cold method'). A result was interpreted as positive for mutagenicity if the clear zone on the streaked line of strain M45 appeared 3 mm or more longer than that on the line of strain H17.

Table 2. *Test samples showing antimicrobial activity* in the* rec-*assay of crude forms of spices and their water-heated and water-macerated filtrates and residues, using* Bacillus subtilis *H17* (rec⁺) *and M45* (rec⁻)

Spice	Standard streak method: *B. subtilis* H17	Standard streak method: *B. subtilis* M45	Standard streak method: Difference	Standard streak method: Rec effect†	Cold method: *B. subtilis* H17	Cold method: *B. subtilis* M45	Cold method: Difference	Cold method: Rec effect†
Crude forms								
Galangal	5	5	0	−	5·5	7	1·5	−
Garlic	41	42	1	−	41	43	2	−
Shallot	1·5	2·5	1	−	6·5	9	2·5	−
Zedoary	0	1·5	1·5	−	2·5	4	1·5	−
Ceylon cinnamon	6·5	14	7·5	+	7	14	7	+
Chinese star anise	0	2	2	−	2	2	0	−
Clove	5	5	0	−	11	9	−2	−
Turmeric	0	0	0	−	2	2	0	−
Wild ginger	4	6	2	−	3	5	2	−
Water-heated residues								
Ceylon cinnamon	17·5	23·5	6	+	20	29·5	9·5	+
Clove	7	6	−1	−	12	14	2	−
Water-heated filtrates								
Chinese star anise (50 mg solids/disc)	1	2	1	−	5	5	0	−
Clove (50 mg solids/disc)	3	2	−1	−	2	2	0	−
Water-macerated residues								
Garlic	7	8	1	−	10	11	1	−
Ceylon cinnamon	6·5	10·5	4	+	11	25	14	+
Clove	11	12	1	−	23	23	0	−
Wild ginger	2	2	0	−	1·5	3	1·5	−
Water-macerated filtrates								
Garlic (50 mg solids/disc)	6	7	1	−	12	11	−1	−
Clove (50 mg solids/disc)	2	1	−1	−	2	3	1	−

Lengths of clear zone (mm)

*The other samples tested (see Table 1) showed no antimicrobial activity in either variant of the assay (standard streak or cold method).
†Indicating no mutagenic activity (−) and with mutagenic activity (+).

RESULTS AND DISCUSSION

The results in Table 2 show that many of the tested spices showed antimicrobial activity against strains H17 and M45. In the crude form, these spices were galangal, garlic, shallot, zedoary, Ceylon cinnamon, Chinese star anise, clove, turmeric and wild ginger. Among these, garlic produced the longest clear zones for both strains, with lengths of about 40 mm by both the standard streak and cold methods. When the spices had been heated with water, only the residues of Ceylon cinnamon and clove retained their antimicrobial activity, while the untreated water-heated filtrates had no such effect. However, when these filtrates were concentrated, Chinese star anise and clove at 50 mg/disc produced some clear zones on the streak lines of H17 and M45. The water-macerated residues of four samples of spices, namely, garlic, Ceylon cinnamon, clove and wild ginger, possessed antimicrobial activity, and again the corresponding filtrates showed no activity until after their concentration to 50 mg/disc, at which point both garlic and clove caused inhibition of both test strains.

When the lengths of the clear zones for strains H17 and M45 were compared, only Ceylon cinnamon had a *rec* effect, indicating the possession of mutagenic activity. This effect was exhibited by the crude form of the spice and also by the water-heated and water-macerated residues, but not by the water-heated and water-macerated filtrates, showing the responsible component(s) to be heat stable but not water soluble. Organic-solvent fractions have not been prepared and it is not yet known whether the fractions showing a mutagenic action are also carcinogenic. Another aspect requiring investigation is the possible influence of metabolic processes on the mutagenic activity of spice constituents, and work is at present in progress on the mutagenicity of various Ceylon cinnamon extracts using the spore *rec*-assay in the presence and absence of a hepatic metabolizing system.

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FORMATION OF MUTAGENS IN BOILED PORK EXTRACT

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Abstract—When boiled pork extract was heated under reflux at 102°C for 4 hr mutagens, which were detected using *Salmonella typhimurium* strains TA98 and TA1538, were formed. The level of mutagenicity was dependent on the concentration of pork in the extract, the duration of boiling and on pH; the optimum pH for mutagen formation was found to be 9 to 11. Thin-layer chromatographic analysis showed that the mutagens formed in boiled pork extract were chromatographically distinguishable from benzo[*a*]pyrene and from the primary mutagenic pyrolysis products of tryptophan (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole and of glutamic acid (2-amino-6-methyldipyrido[1,2-*a*:3',2'-d]imidazole).

INTRODUCTION

Since 1964, carcinomas of the liver and stomach have together constituted the second commonest cause of death among the Chinese in Taiwan. To study the aetiological factors contributing to these malignant tumours the Ames test has been used to detect mutagens in extracts of the foods that are most popular with the Chinese. In preliminary investigations we found that ether extracts of soya bean sauce and Chinese wine contain the precursors of mutagens (Lin & Tai, 1980; Lin, Wang & Yeh, 1979).

The active mutagenic components were recently isolated from the pyrolysis products of certain amino acids (e.g. tryptophan and glutamic acid) and their structures have been characterized (Kasai, Nishimura, Nagao *et al.* 1979; Matsumoto, Yoshida, Mizusaki & Okamoto, 1977; Yamamoto, Tsuji, Kosuge *et al.* 1978).

Mutagenic activity has been found in boiled beef extracts and concern has also arisen since the Chinese in Taiwan usually cook ground pork with soya sauce for a relatively long time. Preliminary results indicated the presence of substances in the boiled pork extracts that were mutagenic when tested with *Salmonella typhimurium* strains TA98 and TA1538 but not with strains TA100, TA1535 and TA1537 (Lee & Lin, 1981). Since a correlation between mutagenicity and carcinogenicity has been demonstrated (McCann, Choi, Yamasaki & Ames, 1975) it is possible that the strong mutagens in boiled pork may be among the factors responsible for diet-related cancers.

We now report a study of mutagen formation during the boiling of pork extract as a function of boiling time, pH and concentration.

EXPERIMENTAL

Materials

Benzo[*a*]pyrene was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The amino-acid pyrolysis products, Trp-P-1 and Glu-P-1 were kindly provided by Dr T. Sugimura, and NADP and glucose 6-phosphate were obtained from Calbiochem (San Diego, CA, USA). The food materials were purchased from local stores in Taipei City.

Sample preparation

Effect of boiling time on mutagen formation. The boiled pork extracts were prepared by boiling 1200 g of lean ground pork in two volumes of distilled water for 20 min. The solid residues were removed by filtration with glass wool and the filtrate was cooled. The congealed fat was skimmed off and the remaining liquid was condensed to 240 ml by boiling in an open beaker. The condensate was boiled under reflux for various periods of time. The sample was kept at 100–102°C during the heating. After heating, the boiled pork extract was acidified with 4 M-HCl to pH 2, saturated with $(NH_4)_2SO_4$, filtered through glass wool and extracted twice, each time with one volume of dichloromethane. The aqueous phase was then adjusted to pH 10 with 4 M-ammonia water and extracted three times each with one volume dichloromethane. The acidic extracts and the basic extracts were evaporated separately to dryness. The residues were weighed and dissolved in dimethylsulphoxide for mutagenicity testing. Since most of the mutagens were found in the basic fraction, the residues obtained from the basic fraction were used in these investigations.

Effect of concentrations of pork extracts on mutagen formation. The boiled pork extracts were prepared by boiling 600 g of lean pork as described above, and the concentrations were adjusted with distilled water to 12, 6, 3 and 1·5 g equivalents fresh ground pork per ml. They were further boiled for 12 hr under reflux and extracted for the mutagenesis assay.

Effect of pH on mutagen formation. The boiled pork extract was prepared by boiling 900 g of lean pork as described above. The final concentration was adjusted to be equivalent to 6 g fresh ground pork per ml. 4 M-HCl or 4 M-ammonium hydroxide were used to adjust the pH of the extracts to 1, 3, 5, 7, 9 and 11, and they were further heated and extracted for the mutagenesis assay.

Abbreviations: Glu-P-1 = Glutamic acid pyrolysis product 1 (2-amino-6-methyldipyrido [1,2-*a*:3',2'-d]imidazole); Trp-P-1 = tryptophan pyrolysis product 1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole).

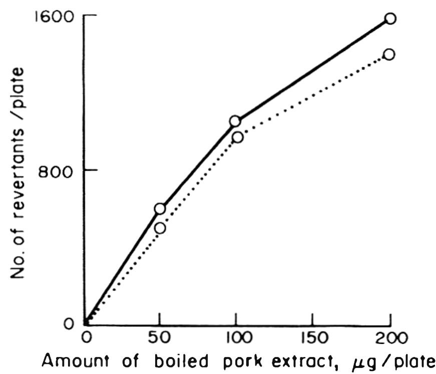

Fig. 1. Dose response curves for the mutagenicity of boiled pork extract tested with *Salmonella typhimurium* strains TA1538 (—O—) and TA98 (···O···).

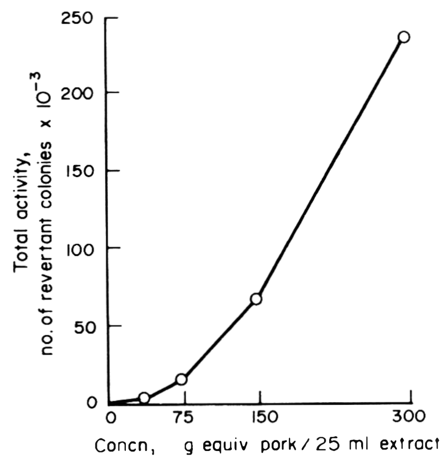

Fig. 3. The effects of different concentrations of ground pork in the extract on the mutagenicity in *Salmonella typhimurium* strain TA1538 of boiled pork extract after refluxing at 102°C for 12 hr.

Mutagenesis assay

Mutagenic activity was measured by the preincubation method as described by Yahagi, Nagao, Seino *et al.* (1977). The liver homogenate supernatant (S-9) was prepared from the liver of male Sprague–Dawley rats (180–200 g) which were treated with Aroclor 1254 as described by Ames, McCann & Yamasaki (1975). *S. typhimurium* strains TA1538 and TA98 were kindly supplied by Professor B. N. Ames (Berkeley, CA). The colonies of histidine revertants on each plate were counted after incubation at 37°C for 2 days. The rates of spontaneous reversion in the presence of S-9 mix were 43 ± 8 and 24 ± 3 colonies/plate for strain TA98 and TA1538 respectively. The numbers of spontaneous revertants were subtracted from the tested group in these experiments.

Thin-layer chromatography

Thin-layer chromatography of boiled pork extracts, benzo[*a*]pyrene or Glu-P-1 was carried out using a glass-filter sheet impregnated with silica gel (Silica gel 60 F-254, E. Merck, Darmstadt, FRG) and chloroform–methanol (90:10, v/v) as the solvent system. The chromatograms were cut into 1-cm strips which were individually extracted. The extracts were dried and dissolved in DMSO, and aliquots were tested with *S. typhimurium* strain TA1538.

RESULTS AND DISCUSSION

Mutagenic activity was detected in ground pork extracts after boiling and the mutagens were specific for strains TA98 and TA1538 and required metabolic activation by S-9 mix (Lee & Lin, 1981). Figure 1 shows the linear dose-response of the two strains that are sensitive to frame-shift mutation. The effects of cooking time on mutagen formation are shown in Fig. 2. The data show a lag phase of 4 hr, during which significant levels of mutagen were not produced, and an active phase during which the mutagenic activity increased rapidly with increased cooking time. The reaction mixture was brown with a light roasted aroma after 4 hr of cooking. The formation of mutagen in boiled beef extracts is much slower with a lag phase of around 48 hr (Spingarn & Weisburger, 1979). The effects of various concentrations of boiled pork extracts on mutagen formation are shown in Fig. 3. It indicates that the minimum concentration of boiled pork extract required to induce mutations is about 3 g equivalents of fresh ground pork per ml of extract. Figure 4 illustrates the effects of pH on mutagen for-

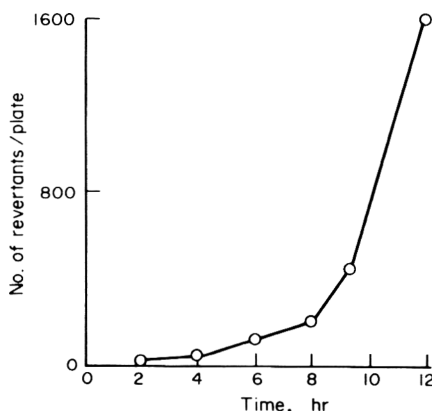

Fig. 2. The effects of boiling time (under reflux at 102°C) on the mutagenicity in *Salmonella typhimurium* strain TA1538 of a boiled pork extract containing 5 g equivalents of fresh ground pork in each ml of extract.

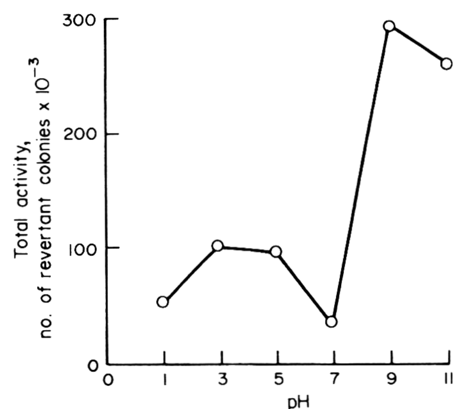

Fig. 4. The effects of pH on the mutagenicity in *Salmonella typhimurium* strain TA1538 of boiled pork extract containing 6 g equivalents of fresh ground pork in each ml extract after refluxing at 102°C for 12 hr.

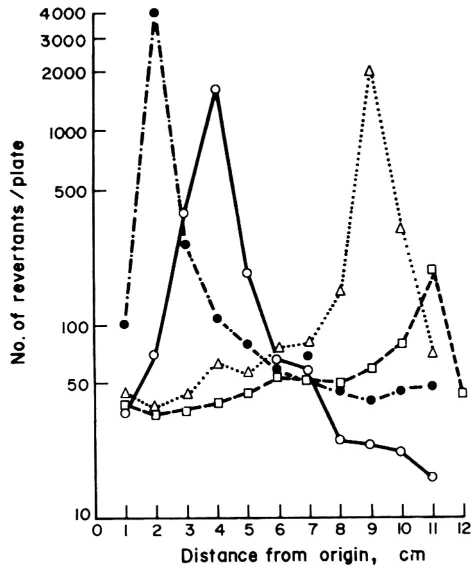

Fig. 5. The results of thin-layer chromatographic analysis of boiled pork extracts (—○—), benzo[*a*]pyrene (- -□- -), tryptophan pyrolysis product 1 (-·-●-·-) and glutamate pyrolysis product 1 (····△····).

mation. The formation of mutagens is enhanced at pH values above 9, and is much less at neutral or acidic pH. This result indicates that the formation of the mutagens in boiled pork is probably not caused by the sugar–ammonia model system proposed by Spingarn & Garvie (1979).

Thin-layer chromatography was used to determine whether the mutagenic activity of the boiled pork extract resulted from the presence of benzo[*a*]pyrene or of the mutagenic amino acid pyrolysis products, Trp-*P*-1 or Glu-P-1. Figure 5 shows that the boiled pork extract has a peak at R_F 0·4, while Trp-*P*-1 has a peak at R_F 0·2, Glu-P-1 at R_F 0·8 and benzo[*a*]pyrene at R_F 1·0. These analyses indicate that the mutagens in boiled pork extract are chromatographically distinguishable from benzo[*a*]pyrene, Trp-P-1 and Glu-*P*-1.

A favourite Chinese dish, 'Ru-thou Fan' is made by cooking ground pork and reducing it to a highly flavoured paste which is served on steamed rice. Our results show that this paste may have strong mutagenic activity and could contribute to the incidence of cancer of the gastro-intestinal tract and other organs.

The purification and identification of the mutagens from the boiled pork extract are in progress.

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INHIBITION OF PROTEIN PYROLYSATE MUTAGENICITY BY RETINOL (VITAMIN A)

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Abstract—The mutagenicity in the Ames Salmonella-microsome test of four protein pyrolysate products, formed during the cooking of meat, (Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2) was found to be inhibited by the addition of vitamin A *in vitro* in the form of retinol. The effect is interpreted as an inhibition of the metabolic activation of the mutagens to their respective ultimate mutagenic forms since retinol has been shown to have no effect on the survival of the Salmonella cells, no effect on directly acting mutagens and no effect on the formation of NADPH in the test system. The results demonstrate the need for an increased understanding of the interaction of dietary components in evaluating mutagenic/carcinogenic risks from processed food.

INTRODUCTION

Short-term tests for mutagenicity, in particular the Ames Salmonella-microsome test, have been used to an increasing extent to screen for potential mutagens and carcinogens in the environment. While the qualitative correlation between mutagenicity in the Ames test and carcinogenicity *in vivo* is fairly good (McCann, Choi, Yamasaki & Ames, 1975), the quantitative correlation is less good (Ashby & Styles, 1978) and is dependent on the multistep nature of carcinogenesis: the initiating genotoxic damage, recognized by the mutagenicity assay, may be changed by e.g. DNA-repair processes and promotor or antipromotor agents. However, the initiation process itself may also be influenced by a number of modifying factors such as, for example, vitamin A.

The proposal that vitamin A may affect the initiating step stems from the observation that retinol acts as a modifier of the mutagenicity of compounds requiring metabolic activation (Busk & Ahlborg, 1980 & 1982a,b).

The processing of foods has been found to produce a number of mutagenic compounds (Sugimura & Nagao, 1979) some of which have been shown to have carcinogenic properties in animal studies (Hosaka, Matsushima, Hirono & Sugimura, 1981; Matsukura, Kawachi, Morino *et al.* 1981). However, these compounds seem to be less carcinogenic than would have been expected from their mutagenicity.

Since diet is regarded as a major contributor to the aetiology of cancer and vitamin A is an essential dietary component ingested in different amounts by different groups in our society we undertook the present investigation to elucidate the possible influence of vitamin A on the mutagenicity of some compounds formed during the cooking of meat.

Abbreviations: AαC = 2-Amino-9*H*-pyrido(2,3-b)indole; Glu-P-1 = 2-amino-6-methyldipyrido(1,2-a:3',2'-d)imidazol; Glu-P-2 = 2-aminodipyrido(1,2-a:3',2'-d)imidazol; Trp-P-1 = 3-amino-3,4-dimethyl-5*H*-pyrido(4,3-b)indole; Trp-P-2 = 3-amino-1-methyl-5*H*-pyrido(4,3-b)indole.

EXPERIMENTAL

Animals. Liver homogenate was prepared from male Sprague–Dawley rats (200 g) obtained from Anticimex, Sollentuna, and maintained on Astra Ewos R3 diet, supplied by Ewos AB, Södertälje, containing 12,000 IU vitamin A/kg. The rats were injected ip with Aroclor 1254 (500 mg/kg) dissolved in arachis oil 5 days before they were killed. The animals were killed by decapitation and the livers were removed aseptically. All subsequent steps were performed under sterile conditions and at +4°C. The tissue was minced and homogenized in three vol. 0·15 M-KCl with four strokes of the pestle at 1000 rev./min in a Potter-Elvenhjelm homogenizer. After centrifugation at 9000 **g** for 10 min the supernatants (S-9 fractions) from five rats were pooled, divided into aliquots, immediately frozen to −90°C, and stored at this temperature prior to the start of the experiments (max 2 months). The protein concentration, measured by the method of Hartree (1972), of the S-9 fraction was 25·5 mg/ml. The final preparation of the metabolizing system (the S-9 mix) was carried out by the method of Ames, McCann & Yamasaki (1975). The amount of cofactors added to each plate was constant although the amount of S-9 fraction was varied.

Chemicals. Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2 were a gift from Professor T. Sugimura, National Cancer Center Research Institute, Tokyo, Japan. Retinol (all trans), NADP and glucose-6-phosphate were obtained from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals were purchased from Kebo Grave, Spånga.

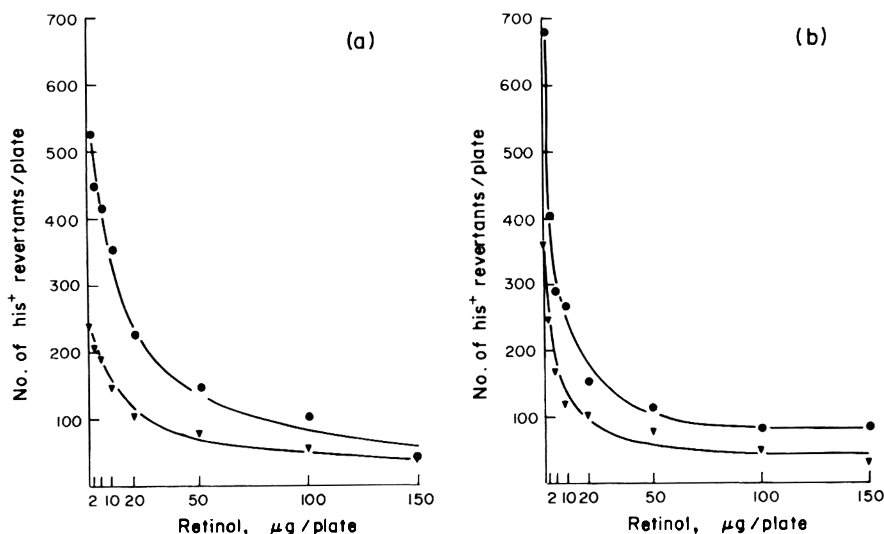

Fig. 1. The mutagenicity of (a) Trp-P-1 at levels of 40 (▼) and 80 (●) ng/plate and (b) Trp-P-2 at levels of 5 (▼) and 10 (●) ng/plate towards *Salmonella typhimurium* strain TA98 in the presence of different amounts of retinol. Each point represents the mean of two experiments with three plates/concentration.

Bacterial tester strain. The *Salmonella typhimurium* histidine auxotroph strain TA98 was kindly provided by Professor B. N. Ames, University of California, USA, and was checked in our laboratory for proper genetic characteristics by the method of Ames *et al.* (1975).

Mutagenicity assay. The procedure of Ames *et al.* (1975) with the modification of Yahagi, Nagao, Seino *et al.* (1977) was followed. The *S. typhimurium* cells were grown for 14 hr at 37°C with continuous shaking. The test compounds were pre-incubated with the S-9 mix at 37°C 20 min prior to the addition of bacteria and top agar. To avoid oxidation, retinol was dissolved in absolute ethanol and added to the liver homogenate from freshly prepared solutions shortly before the addition of the S-9 mix to the test compounds. All retinol solutions were kept on ice and in the dark. The final concentration of ethanol (0·6%) was the same in all S-9 mixes. The addition of ethanol in concentrations varying from 0–1·0% did not affect the mutagenicity of any of the pyrolysate products under the experimental conditions used. The test compounds were dissolved in water and added to the plates in 100 µl portions.

Measurement of NADPH generation. The S-9 mix was prepared as described for the mutagenicity assays with 10 µl liver homogenate/ml. The rate of NADPH generation was measured as an increase in absorbance at 340 nm in a Beckman M24 spectrophotometer. The blank contained the complete reaction mixture with the exception of glucose-6-phosphate. Retinol as a freshly prepared ethanol solution was added to the reaction mixture and the blank as described for the mutagenicity assays.

RESULTS

The mutagenic potency of amino acid pyrolysates in the Ames assay is known to vary with the amount of S-9 added to the plates. The optimal amounts of S-9 for all four test compounds were determined in order to conduct the inhibition experiments in a system with maximal sensitivity. The optimal amounts of S-9 were found to be 8, 10, 15 and 30 µl for Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2, respectively. To elucidate the effect of vitamin A on the mutagenicity of the four amino-acid pyrolysates, vitamin A, in the form of retinol, was added to the test system at doses ranging from 2 to 150 µg/plate.

Figure 1 shows that retinol strongly inhibits the mutagenicity of Trp-P-1 and Trp-P-2 towards strain TA98. Both compounds were tested at two different concentrations, Trp-P-1 at 40 and 80 ng/plate and Trp-P-2 at 5 and 10 ng/plate. The inhibition was readily detected even at the lowest concentration of the vitamin, 2 µg/plate, regardless of the dose of the mutagen and at concentrations of 50–100 µg retinol/plate the inhibition was almost complete. The spontaneous mutation frequency was 25 ± 5 (mean $\pm$ SEM) revertants/plate. Similar results were obtained with Glu-P-1 at concentrations of 7·5 and 15 ng/plate and Glu-P-2 at 500 and 1000 ng/plate (Fig. 2). The inhibition was evident at 2 µg retinol/plate and was almost complete at 50–100 µg retinol/plate.

As different amounts of S-9 were used for the detection of mutagenicity of the various amino acid pyrolysates, the inhibition curves in Figs 1 and 2 are not directly comparable. In Fig. 3, the Ames test data from the highest dose of each mutagen expressed as his$^+$-revertants/plate are plotted against the amount of retinol per mg protein to facilitate comparison of the inhibition at a constant ratio between retinol and the metabolizing enzymes.

To find out whether the inhibition by retinol was due to a depletion of NADPH, a cofactor generated in the test system and vital for the activity of the metabolizing enzymes, the formation of NADPH in the S-9 mix was recorded in the presence of different amounts of retinol. Table 1 shows that retinol at levels of up to 820 µg/mg protein had no effect on the formation of NADPH. The rate of formation varied between 95 and 100% of the control with no added retinol. The highest dose of retinol used in the inhibition experiments was about 750 µg/mg protein (Fig.

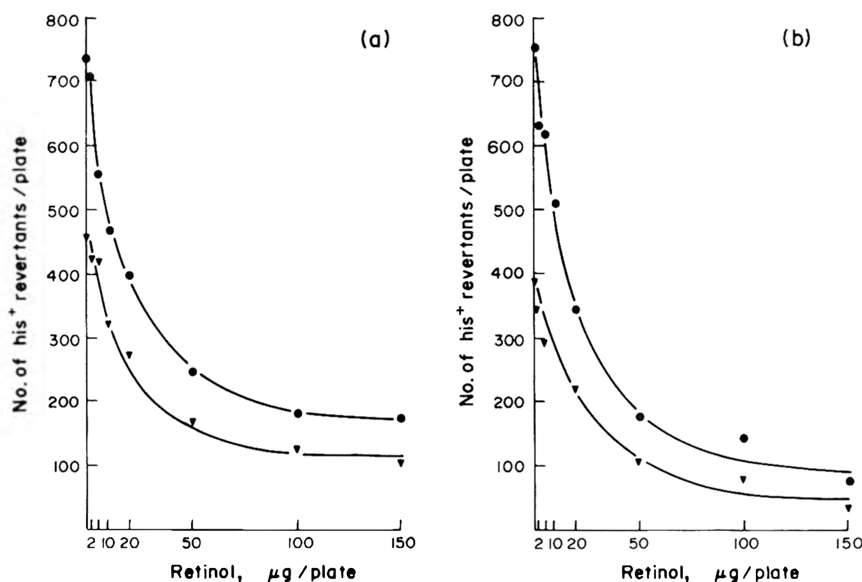

Fig. 2. The mutagenicity of (a) Glu-P-1 at levels of 7·5 (▼) and 15 (●) ng/plate and (b) Glu-P-2 at levels of 500 (▼) and 1000 (●) ng/plate towards *Salmonella typhimurium* strain TA98 in the presence of different amounts of retinol. Each point represents the mean of two experiments with three plates/concentration.

3). However, when retinol was tested, at 1536 µg/mg protein, a 24% reduction in NADPH formation was recorded. The availability of NADPH does not appear to be responsible for the recorded inhibition of mutagenicity under the experimental conditions used.

DISCUSSION

The present data demonstrate that retinol inhibits the mutagenic effect of a series of amino acid pyrolysates. This inhibition is not due to a cytotoxic effect of retinol on the Salmonella cells (Baird & Birnbaum, 1979; Busk & Ahlborg, 1980). It has also been demonstrated that retinol does not inhibit the mutagenic effects of direct-acting mutagens (Baird & Birnbaum, 1979; Busk & Ahlborg, 1980).

As in earlier studies with the indirect mutagens aflatoxin B_1 (Busk & Ahlborg, 1980) and *o*-aminoazotoluene (Busk & Ahlborg, 1982b) the inhibitory effect of retinol presented here is interpreted as the result of an inhibition of the metabolic activation of the test compounds.

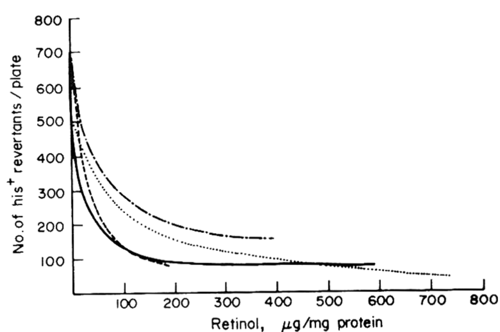

Fig. 3. The mutagenicity of 80 ng Trp-P-1 (·····), 10 ng Trp-P-2 (——), 15 ng Glu-P-1 (—·—) and 1000 ng Glu-P-2 (– – –) towards *Salmonella typhimurium* strain TA98 in the presence of different relative concentrations of retinol and S-9-mix protein.

The metabolism of the pyrolysis products of tryptophan and glutamic acid has not been elucidated in detail. The results of Yamazoe, Ishii, Kamataki *et al.* (1980) suggest that, in the case of Trp-P-2, *N*-hydroxylation is the first step in the metabolic activation and is followed by oxidation to a nitrosocompound. Both the *N*-hydroxy- (M3) and the nitroso- (M4) metabolites (see Fig. 4) are direct-acting mutagens, but recent studies suggest that further conjugation reactions with serine also produce active metabolites (Yamazoe, Ishii, Mita *et al.* 1981). It is therefore difficult to assess which step(s) in the activation of the pyrolysis products are affected by retinol. The proposal that *N*-hydroxylation is inhibited by retinol is supported by our own finding that retinol also inhibits the mutagenicity of *o*-aminoazotoluene (Busk & Ahlborg, 1982b), which is believed to be metabolized via an *N*-hydroxylation (Ames, Durston, Yamasaki & Lee, 1973; Sugimura, Sato, Nagao *et al.* 1976). However we have also found that, under the conditions described in this paper, retinol enhances the mutagenicity of two aromatic amines, 2-aminofluorene and 2-acetylaminofluorene (Busk & Ahlborg, 1982a); the metabolism of both of these compounds is known to involve *N*-hydroxylation as the first step (Aune, Dyb-

Table 1. *The generation of NADPH in the S-9 mix as a function of added retinol*

Retinol added (µg/mg protein)	NADPH generated (nmol/min mg protein)	NADPH generated (% of control)
0	333	100
82	316	95
205	321	96
410	332	100
820	323	97
1536	253	76

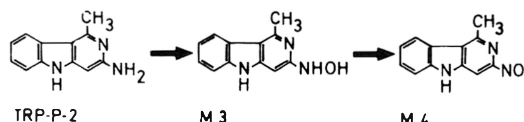

Fig. 4. Proposed metabolic activation of Trp-P-2.

ing & Thorgeirsson, 1980; Sakai, Reinhold, Wirth *et al.* 1978).

A number of epidemiological studies have indicated that vitamin A may act as an anticarcinogenic agent in man (Basu, Donaldson, Jenner *et al.* 1976; Bjelke, 1975; Mettlin & Graham, 1979; Shekelle, Kiu, Raynor *et al.* 1981; Wald, Idle & Boreham, 1980). The mechanism is not known but it has been suggested that promotion could be influenced by the vitamin (Sporn & Newton, 1979). Our present data further supports the hypothesis that vitamin A can also affect initiation.

A number of factors other than vitamin A have been found to modify the mutagenicity of amino acid pyrolysates. Borek & Ong (1981) have shown that X-rays have a synergistic effect on the oncogenic action of Trp-P-2 on golden hamster embryo cells. Yoshida, Matsumoto & Okamoto (1979) found that amino-α-carboline (AαC) had a synergistic effect on Trp-P-2 induced mutagenesis in Salmonella. Pyrrole pigments such as hemin, biliverdine, chlorophyllin and protoporphyrin act as inhibitors of the mutagenicity of some amino acid pyrolysates as shown by Aromoto, Ohara, Namba *et al.* (1980). Tsuda, Takahashi, Nagao *et al.* (1980) presented evidence that nitrite in acidic solution deaminates Trp-P-1, Trp-P-2 and Glu-P-1 and thereby prevents the formation of the ultimate mutagenic forms of these substances. They have also shown that nitrite converts AαC to a non-mutagenic hydroxy derivative but that prolonged nitrite treatment gave a 2-hydroxy-3-nitro- compound that was a direct-acting mutagen (Tsuda, Nagao, Hirayama & Sugimura, 1981a). Inoue, Morita & Kada (1981) have isolated and purified an inhibitor of Trp-P-2 mutagenicity, probably a haemo protein, from cabbage and Hayatsu, Arimoto, Togawa & Makita (1981) have shown that ether extracts of human faeces can inhibit the mutagenicity of Trp-P-1, Glu-P-1 and AαC. The inhibitors in the faeces extracts were found to be oleic and linoleic acid. Furthermore Tsuda, Wakabayashi, Hirayama *et al.* (1981b) have shown that chlorinated tap water strongly inhibits the mutagenicity of both Trp-P-1 and Trp-P-2.

The occurrence of both endogenous and exogenous compounds that can modify the mutagenicity of amino-acid pyrolysates makes it difficult to assess the extent to which they pose a health hazard to man. However, the mere existence of them, in food ingested by man, and their biological activity in model test systems, make them highly suspect as a group of compounds that may be involved in the observed relationship between diet and cancer incidence.

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TOXICOLOGICAL EVALUATION OF COMPOUNDS FOUND IN FOOD USING RAT RENAL EXPLANTS

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Abstract—A rat kidney explant system was developed and its respiratory characteristics in the presence and absence of a series of compounds found in food were studied. Cubes taken from the whole kidneys of male Osborne–Mendel rats were incubated under sterile conditions for up to 18 hr. The respiratory activity of these explants after incubation was measured by determining the amount of $^{14}CO_2$ evolved as a result of the metabolism of [^{14}C]glucose. Changes in membrane permeability as a result of treatment were assessed by measuring the total protein content in the medium. Measurements of respiratory activity showed that the biochemical integrity of the control explants was well maintained over the test period. Various compounds were tested at a range of concentrations from 0·1 to 1·0 mM. In the initial screen, several of the compounds tested at 1 mM—butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), DDT, polychlorinated biphenyl (Aroclor 1254), patulin, zearalenone and cadmium chloride—markedly inhibited respiration; neither ascorbic acid nor caffeine was effective at this concentration. Two of the fat-soluble compounds, DDT and zearalenone, inhibited respiration at concentrations of 0·1 and 0·25 mM concentrations, respectively, while patulin, a water-soluble mycotoxin, did not give significant inhibition at concentrations below 0·5 mM. In some cases, e.g. patulin and BHA, the dose-response curves appeared to be bimodal, with stimulation of respiration occurring at lower concentrations. The two mycotoxins, patulin and zearalenone, and the antioxidant BHA produced losses of cellular protein at concentrations of 1·0, 0·50 and 1·0 mM, respectively, indicating membrane perturbation. Thus, this kidney explant system responded consistently to a variety of known toxins and can be considered as another *in vitro* method of determining which compounds show the potential for *in vivo* toxicity.

INTRODUCTION

The introduction of new substances into commercially prepared food, together with increased consumer interest in the safety of food additives, has led to a greater level of toxicity testing of food components than ever before. New assessment methods are needed to supplement the animal feeding studies that are at present the principal methods of toxicological evaluation. Efforts to develop new methods have centred around the development and validation of *in vitro* procedures, some of which use tissue slices, tissue fractions or isolated cell organelles for short exposure periods (1–2 hr). Somewhat longer periods of exposure have been used for primary or secondary cultures of isolated cells. Renal *in vitro* systems have involved the use of selected cell types (Basačková, 1963) or slices (Berndt, 1976), the latter being suitable for only brief incubation periods.

Sullivan, Chin & Carpenter (1972) developed methods using liver explants to study the metabolism of xenobiotic substances; we have adapted these methods to kidney explants. This approach was chosen because renal explants maintain their cellular architecture and the relative proportions of different cell types while retaining good viability as measured by their capacity for respiration.

The effect of a number of food additives, contaminants and natural components of food on glucose metabolism in renal explants is reported. Some of these substances have also been subjected to extensive biological testing with *in vitro* and/or *in vivo* test systems and have been found to have diverse chemical, physical and toxicological characteristics.

EXPERIMENTAL

Chemicals. Ascorbic acid, BHA, BHT and EDTA were obtained from ICN Pharmaceuticals, Cincinnati, OH, and caffeine and Tween-80 were obtained from Sigma Chemical Co., St. Louis, MO. These chemicals were of the highest purity obtainable from commercial sources and were all used without further purification. Technical DDT was obtained from Chemical Compounding Corp., Brooklyn, NY. Patulin was purified, crystallized and analysed by the Division of Chemistry and Physics, FDA and was found to be 98·7% pure by UV absorption, TLC, HPLC and infrared and mass spectroscopy. Zearalenone was obtained from Commercial Solvents Corp., Terre Haute, IN and was also found to be 98–99% pure by TLC, HPLC and gas–liquid chromatography. The polychlorinated biphenyl used was Aroclor 1254 (Monsanto Co., St. Louis, MO) which had been characterized by the Division of Chemistry and Physics, FDA. Cadmium chloride (reagent grade) was

Abbreviations—BHA = Butylated hydroxyanisole; BHT = butylated hydroxytoluene; CV = coefficient of variation; DDT = 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; EDTA = ethylenediaminetetraacetate; FDA = Food and Drug Administration; HPLC = high-pressure liquid chromatography; SD = standard deviation.

obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. [^{14}C]Glucose (uniformly labelled, specific activity c. 4 mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA.

Explant technique. Male Osborne–Mendel rats weighing 300–350 g were killed by decapitation, and their kidneys were removed aseptically. In a typical experiment requiring 40 cultures, the kidneys of four rats were used. The kidneys were halved longitudinally and cut in 0·5 mm slices on a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Gomshall, Surrey, England). After a single pass through the machine, the cutting platform was rotated through 90° and a second cutting was executed. The cross-sectional dimensions of the resulting rectangular prisms of tissue or 'tissue cubes' were 0·5 × 0·5 mm (× 1/2 the kidney thickness). Each explant comprised the relative amounts of medulla and cortex appropriate to its site of origin in the organ. The kidney explants were placed in sterile Dulbecco's phosphate-buffered saline (Dulbecco & Vogt, 1954) containing 100 units of penicillin and 100 μg of streptomycin/ml. All kidney cubes were placed in the same container of saline; this procedure randomizes the explant material used in each culture dish with regard to the donor animal and the site of origin in the kidney. Pooling explant material from several animals increases the reproducibility of replicates, both within and between experiments (Hirsch, 1976). The pH of the culture medium after incubation (measurement made in a 5% CO_2 atmosphere) was within 0·2 units of the initial pH of 7·4.

The tissue was removed from the saline and 250-mg quantities of tissue were placed in 60-mm Falcon plastic petri dishes. Trowell's T-8 medium (2·5 ml) (Trowell, 1959) containing penicillin and streptomycin was added to each dish; the medium covered the bottom of the dish but was not deep enough to submerge the tissue completely. Preliminary experiments demonstrated that the addition of foetal calf serum to the medium at levels of up to 20% did not have a significant effect on the glucose utilization of the cultures over an 18-hr incubation period; therefore the serum was not added in further experiments.

The prepared dishes were placed in 26-cm plastic desiccators (Ace Duravac, Ace Glass Co., Vineland, NJ) which were modified by the addition of tubes at the bottom to facilitate flushing with gas. The desiccators were flushed for 15–20 min with 95% O_2–5% CO_2 and then placed in an incubator at 37°C. After the cultures had equilibrated for 1 hr, test compounds dissolved in 100 μl of either distilled water or dimethylsulphoxide (DMSO) were added. The appropriate solvent alone was added to control cultures. The desiccators were again flushed with the O_2–CO_2 mixture for 10 min and then returned to the incubator for 18 hr. Most experiments consisted of eight replicates (eight dishes) at each dose level.

Respiration measurements. After exposure of the cultures to the test compound, the entire contents of the dishes were transferred to conical tubes and centrifuged at 600 **g** for 10 min. The medium was decanted and reserved for the measurement of protein. Buffered saline (2·5 ml), which had been saturated with 95% O_2–5% CO_2, was then added, the tissue was gently suspended and the centrifugation was repeated. The saline was decanted and discarded. The tissue was then transferred to a 25-ml Erlenmeyer flask, and 2·5 ml buffered saline was added to the flask followed by 100 μl 0·9 M-glucose solution containing 2·5 μCi [^{14}C]glucose. The flasks were closed with serum stoppers into which plastic centre wells (No. K-882320, Kontes Co., Vineland, NJ) had been inserted and placed in a reciprocating water bath (100 strokes/min) at 37°C for 60 min. At the end of the incubation period, 0·2 ml NCS tissue solubilizer (Amersham/Searle, Arlington Heights, IL) was placed in the centre well of each vessel for the collection of evolved $^{14}CO_2$ and 10% H_2SO_4 (0·25 ml) was added to the medium. The flasks were then returned to the water bath for a further 30 min after which the centre wells were removed and placed in counting vials. The absorbed radioactivity was counted in a Model 6880 Mark III liquid scintillation counter (Searle Radiographics Inc., Des Plaines, IL), using Permafluor I scintillation fluid (Packard Instrument Co., Downers Grove, IL). Counting efficiency was approximately 95% and counting time was adjusted to allow for a probable counting error of less than 1%. Counts were converted to dpm using stored quench curves and an internal microprocessor. The explant material was collected on tared filter paper disks, rinsed, dried overnight and weighed. The $^{14}CO_2$ produced was expressed at dpm/mg dry weight of tissue/hr.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as the standard. Statistical analyses were performed using the IBM Statistical Library Program for APL-IUP 5796-pgq (IBM Corp., Inc., Advanced Logic Products, Poughkeepsie, NY). Differences between means were analysed by the Student's *t* test, and *P* values of $\leq 0·05$ were accepted as statistically significant.

RESULTS

Because five of the compounds (BHA, BHT, PCB, zearalenone and DDT) were poorly soluble in water, they were dissolved in DMSO. Control cultures exposed to up to 100 μl DMSO/2·5 ml culture volume showed no statistically significant effect on respiration. This is consistent with the findings of Metcalfe (1971), who concluded that levels of DMSO up to about 1% have negligible effects on primary cultures of monkey kidney cells.

Initial studies were carried out with untreated cultures to establish the time course of $^{14}CO_2$ production from glucose by the kidney explants; the data shown in Fig. 1 represent several similar experiments. The rate of respiration declined for the first 10 hr and then increased abruptly, with glucose utilization reaching a maximum value 14 hr after establishment of the explants. If the explants were held for several days, respiration diminished by about 20% in each 24-hr period.

Table 1 shows the mean respiration values for replicate control cultures in eight of the experiments reported here; the data were obtained over a 2-year period. The standard deviations (SD) and coefficients of variation (CV: [SD/mean] × 100) for each experiment are also shown. The within-run reproducibility of the method is reflected in the CV, which for all

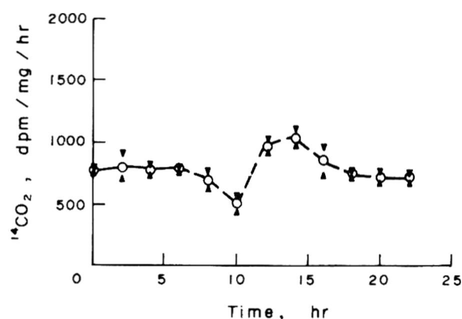

Fig. 1. The rate of respiration of rat kidney explants as measured by $^{14}CO_2$ production with [^{14}C]glucose as substrate. Values are the means of four determinations at each time period and the triangles indicate standard errors of the mean (SEM).

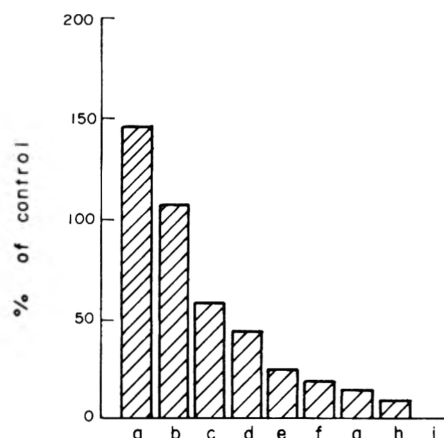

Fig. 2. The rates of respiration of rat kidney explants, as measured by the production of $^{14}CO_2$ with [^{14}C]glucose as substrate, after 18 hr incubation with 1 mM solutions of (a) ascorbic acid, (b) caffeine, (c) BHT, (d) DDT, (e) PCB, (f) patulin, (g) BHA, (h) zearalenone and (i) $CdCl_2$. The results represent the combined data from eight separate experiments. Except for PCB and $CdCl_2$, where duplicate determinations were carried out, six to eight replicates were used for each of the test compounds and corresponding controls.

eight experiments averaged 25. The means of all the control means and SDs for the eight experiments were 828 and 205, respectively (CV = 24·8).

Figure 2 summarizes the effect of various chemicals at 1 mM concentrations on the respiration of kidney explants after an 18-hr exposure. Ascorbic acid increased respiration above control levels and BHT, DDT, PCB, patulin, BHA, zearalenone and cadmium chloride reduced respiration. Caffeine had no effect.

The effects of different concentrations of BHA, BHT and ascorbic acid, caffeine, patulin, zearalenone and DDT on respiration and protein leakage are shown in Fig. 3. In cultures incubated with 0·10, 0·25 and 0·50 mM-BHA and with 0·10 mM-BHT, respiration was increased over control values (Figs 3a,b), but only the increases with 0·10 and 0·50 mM-BHA were statistically significant. Inhibition occurred at the higher concentrations of both BHA and BHT, and was statistically significant with 0·75 and 1·0 mM-BHA and with 1·0 mM-BHT. Ascorbic acid had no effect at any concentration tested (Fig. 3c). Leakage of protein into the medium in the presence of these compounds was statistically significant only with 1·0 mM-BHA. Caffeine had no pronounced effects on respiration; glucose utilization was slightly stimulated at the 0·50 and 1·0 mM concentrations but the increases were not statistically significant (Fig. 3d). Protein content of the medium was significantly decreased with both 0·50 and 1·0 mM-caffeine. Respiration was significantly increased in cultures incubated with 0·10 mM-patulin but was significantly decreased in those incubated with the 0·50, 0·75 and 1·0 mM concentrations (Fig. 3e). Protein leakage was significantly increased at 1·0 mM-patulin, and at this concentration respiration of the explants had almost ceased. Zearalenone (Fig. 3f) produced a pronounced dose-related inhibition of kidney explant respiration. Other than cadmium chloride (Fig. 2), zearalenone was the most effective compound tested in this study in inhibiting glucose metabolism. At 0·25 mM-zearalenone, respiration was inhibited by approximately 70%. In addition, treatment with this compound increased protein loss from the tissue relative to that seen for the control cultures by about 35% at the two highest concentrations tested. DDT (Fig. 3g) exhibited a dose-response simi-

Table 1. *Mean values and variances of respiration of control cultures of kidney explants as indicated by the level of $^{14}CO_2$ produced with [^{14}C]glucose as the substrate*

Experiment no.	No. of replicates	Specific activity of $^{14}CO_2$ released (dpm/mg tissue/hr)	SD	CV*
1	8	769	116	15
2	6	397	157	39
3	8	1018	123	12
4	8	791	198	25
5	8	560	131	23
6	8	899	293	32
7	8	718	157	21
8	6	1468	465	31

SD = Standard deviation
CV = Coefficient of variation, (SD/mean) × 100

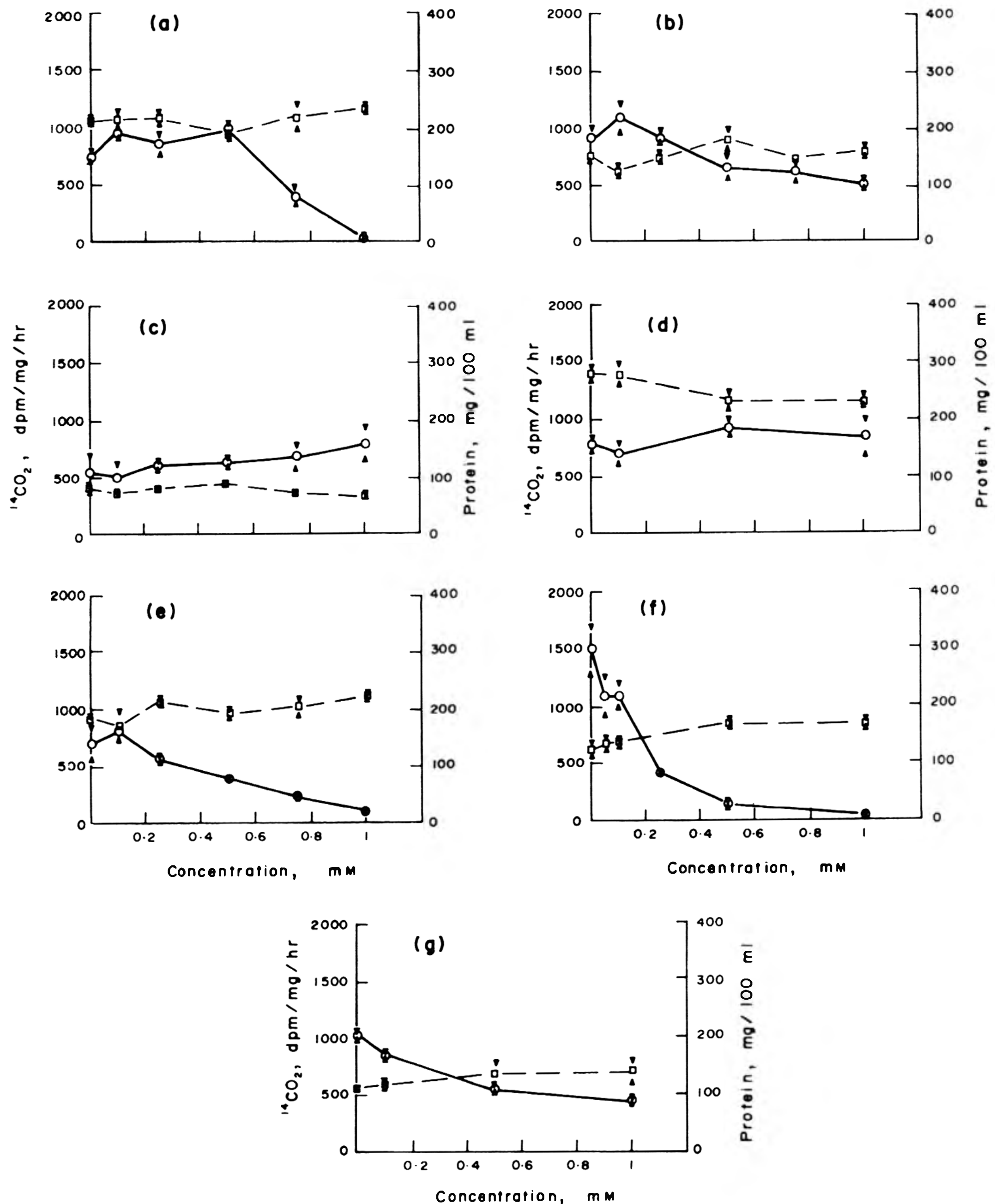

Fig. 3. The effects, on the respired $^{14}CO_2$ (——) produced by rat kidney explants with [^{14}C]glucose as the substrate and on the appearance of protein in the medium (---), of treatment with (a) BHA, (b) BHT, (c) ascorbic acid, (d) caffeine, (e) patulin, (f) zearalenone and (g) DDT. Values are means ± SEM of eight replicates.

lar to that seen for BHT. At the 0·50 and 1·0 mM concentrations, the inhibitory activity was 46 and 56%, respectively. Little protein leakage was noted.

DISCUSSION

We have studied the respiration of the kidney explant system in the presence and absence of a series of test compounds. By modifying the liver explant system of Sullivan *et al.* (1972) we were able to maintain kidney preparations in a favourable milieu. In this system, kidney explants could be exposed to test compounds for significantly longer times than is possible when tissue slice techniques are used, without disrupting the anatomical relationships of the cells (Berndt, 1976).

In general, tissue integrity was maintained for the 18-hr period. This assessment was based on measurement of the respiration rate of control cultures. Reproducibility of respiration measurements within any series of cultures was good; the CV was generally

around 25 for control cultures and this degree of variation was consistent from experiment to experiment.

The compounds tested ranged from known potent toxins with anticipated effects to compounds with little reported cellular toxicity; they are all contaminants or natural components of food or food additives. Their effect on the kidney explants was assessed primarily on the basis of total respiration as measured by the conversion of ^{14}C-labelled glucose to $^{14}CO_2$. Because of the implications of positive findings with respiration, particularly at low concentrations of the test material, we selected respiration as the criterion for investigating damage to the metabolic integrity of the explants.

The use of T-8, a serum-free medium, permitted the measurement of leakage of total protein, or Lowry-positive material, from the cells. This parameter proved to be a much less sensitive indicator of compound-cell interaction than the rate of glucose utilization. Only a few of the compounds elicited a significant effect on protein leakage, and these changes occurred only at relatively high concentrations. Patulin was one of those compounds, a finding in accordance with other reports reviewed by Singh (1967) indicating an effect of this compound on the semipermeability of cell membranes. Patulin was an effective inhibitor of glucose oxidation in the kidney explants, an effect not unexpected since Singh (1967) reported that respiration of several tissues and cellular systems is inhibited by this toxin. A more recent report (Polacco & Sands, 1977) demonstrated that 0·062 mM-patulin caused 50–60% inhibition of respiration in cultured soya-bean suspensions. This concentration of toxin was approximately one-tenth of the amount needed to produce an effect of similar magnitude in the kidney explant system. Liver slices are also susceptible to inhibition of respiration by patulin at 0·08–0·32 mM concentrations (Peters *et al.* 1977). In preliminary experiments, we confirmed the observations of Ciegler (1977) that patulin may exert its toxicity by interaction with −SH groups, since addition of cysteine to the medium attenuated the effect of the toxin on kidney culture respiration.

The other mycotoxin tested, zearalenone, has little similarity to patulin with regard to chemical or physical properties, but it was also effective in retarding the respiratory activity of the kidney preparations. Aside from its known oestrogenic activity (Mirocha *et al.* 1977), very little has been reported about the biological activity of this compound. A recently completed chronic study in which rats were fed zearalenone at levels as high as 3 mg/kg body weight for approximately 2 yr revealed that the toxicity of this compound is low and no specific tissue lesions were reported except for an increase in the prominence of medullary trabeculae (Cox & Re, 1979). It may be speculated that the mode of action was nonspecific interference with intracellular membrane functions, as has been reported for other lipophilic substances (Metcalfe, 1971). Caffeine, another natural compound which is not known to produce any specific tissue lesions *in vivo* except at very high acute oral doses (Friedman *et al.* 1979), did not interfere with glucose oxidation in the kidney cultures.

The inhibition of glucose metabolism by DDT may be related to its lipophilicity. This compound has also been reported to be cytotoxic to mammalian cells *in vitro* at even lower concentrations than those reported in the present studies. Palmer, Green & Legator (1972) showed that DDT was toxic to cultured kangaroo rat cells at concentrations of 20–50 μg/ml. The reduced production of $^{14}CO_2$ from [^{14}C]glucose by kidney explants might have been predicted, since Johnston (1951) reported that 0·01–0·1 mM-DDT produced inhibition of succinoxidase and cytochrome oxidase, two key respiratory enzymes.

Heavy metals such as cadmium are thought to exert their toxic effects through specific cell-membrane lesions. In the present study, 1 mM-cadmium chloride almost totally destroyed the respiratory activity of the kidney explants. The effect was apparently not specific to cadmium since 2·5 mM-zinc sulphate also markedly depressed respiration of the kidney cells (data not shown).

Aroclor 1254 at a concentration of 1 mM produced impairment of kidney cell respiration but to a lesser degree than that brought about by the cadmium salt. Aroclor 1254 produced impairments in a wide variety of biochemical functions when fed to rats; these changes included an apparent uncoupling of liver mitochondrial oxidative phosphorylation (Garthoff, Friedman, Farber *et al.* 1977). This latter effect and the nonpolar nature of the chlorophenolic isomers constituting Aroclor 1254 raises the possibility that the biological activity of PCB may be due to membrane involvement such as structural perturbation.

Although both BHA and BHT are known to possess low acute toxicity (Feuer, Gaunt, Golberg & Fairweather, 1965; Gaunt, Gilbert & Martin, 1965), the series of studies reported here confirm the results of others (Leslie *et al.* 1978; Metcalfe, 1971; Milner, 1967; Surak, Bradley, Branen & Shrago, 1976a; Surak, Bradley, Branen *et al.* 1976b) concerning the biological and biochemical activities of these compounds in *in vitro* systems. Much evidence has also been presented to show that the biological effects of these two phenolic antioxidants relate, at least partially, to their nonpolar character and consequent apparent ability to react and bind with membranes, altering their physical and possibly functional properties (Leslie *et al.* 1978; Metcalfe, 1971; Snipes, Person, Keath & Cupp, 1975; Surak *et al.* 1976a,b; Surak, 1980). As in most of these *in vitro* studies and in short-term feeding studies (Feuer *et al.* 1965) of both compounds, in our study BHT appeared to have a more profound effect than BHA on the kidney explants (Figs 3a,b), that is it inhibited respiration at lower concentrations than BHA. Moreover, both compounds appeared to stimulate cellular respiration in our system at low concentrations (0·1 mM) in a highly reproducible manner. This phenomenon may be related to the finding of Surak (1980) that low levels of BHA and BHT had a membrane-stabilizing effect on erythrocytes, while high levels caused haemolysis in treated animals. Evidence for BHA/BHT-related disturbance of membrane integrity in the cells of the kidney explants was apparent from measurements of protein in the medium (Figs 3a,b). Increases of protein in the medium occurred with both compounds, but were significant only for BHA and only at the highest level tested.

The possibility that the antioxidant activity of BHA

and BHT is responsible for their inhibition of respiratory activity in the kidney explants appears unlikely because similar concentrations of ascorbic acid were ineffective. Milner (1967) also showed that tocopherol did not mimic the effect of BHT in inhibiting RNA synthesis by monkey kidney cells. The relatively low toxicity of BHA (and possibly BHT) *in vivo* may be due to its rapid metabolism and excretion (Hathway, 1967).

Concentrations as high as 1 mM were sometimes required for the inhibition of respiration. However, lower levels often produced a stimulatory effect and this finding should be included in the assessment of the toxicity of the compounds to the explants. This is especially important in those cases in which the effect may be related to an uncoupling of oxidative phosphorylation (Wainio, 1970). Cadmium chloride, for example, significantly stimulated respiration of the explants at a concentration of 10^{-7} M in preliminary experiments.

In conclusion, we have used model toxic compounds to demonstrate the potential of the kidney explant system for assessing the effects of xenobiotic substances. The system is relatively simple and responds predictably to compounds that affect the biochemical integrity of tissues, and the rate of respiration of the explants is maintained for periods of time that permit longer exposure to the compounds than do tissue-slice techniques. In addition, it may be possible to measure effects of compounds on specific renal function with this system.

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HIGH INCIDENCE OF ANGIOSARCOMAS IN BROWN-FAT TISSUE AND LIVERS OF MICE FED STERIGMATOCYSTIN

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Abstract—BDF_1 female mice fed 30 or 120 ppm sterigmatocystin in their diet for 55 or 51 wk, respectively, developed angiosarcomas of the liver and of the dorsal brown-fat tissue. No tumours were observed in five mice from each group, including the controls, killed after 43 wk, but angiosarcomas were observed in mice from the test groups that died or were killed after more than 43 wk of treatment with sterigmatocystin. Thirty-four out of 53 mice (64·2%) fed 30 ppm sterigmatocystin developed hepatic angiosarcomas. Six (11·3%) of those fed 30 ppm sterigmatocystin and 27 out of the 51 mice (52·9%) fed 120 ppm developed angiosarcomas in the dorsal brown-fat tissue. Benign vascular lesions in the liver and a few angiosarcomas of the ovary and lung were also observed in mice of both test groups. No vascular changes were observed in control mice except for peliosis-like lesions in the spleens of two mice. Increases in the incidences of lung and hepatocellular adenomas were also observed in sterigmatocystin-treated mice.

INTRODUCTION

Sterigmatocystin, a mycotoxin produced by several species of Aspergillus, has been shown to be a potent carcinogen in the rat (Mabuchi, 1979; Maekawa, Kajiwara, Odashima & Kurata, 1979; Ohtsubo, Saito, Kimura & Tsuruta, 1978; Purchase & van der Watt, 1970) and in a small aquarium fish, medaka (Hatanaka, Doke, Harada *et al.* 1981) and to manifest "genetic activity" in bacteria (Ueno, Kubota, Ho & Nakamura, 1978; Wehner, Thiel, van Rensburg & Demasius, 1978) and mammalian cells in tissue culture (Umeda, Tsutsui & Saito, 1977). A large number of fungi have been found to produce sterigmatocystin (Rabie, Lübben & Steyn, 1976) and recent analyses carried out in Japan have frequently revealed both domestic and imported cereals to be contaminated with these fungi (Tsuruta, 1980).

We have recently reported that in medaka fed 2·5 ppm sterigmatocystin in the diet for 8 wk there was a high incidence of liver-cell carcinoma within 18 wk (Hatanaka *et al.* 1981). Guinea-pigs were very sensitive to the toxicity of sterigmatocystin, but showed no tumour development (Mabuchi, 1979). Mice are known to be resistant to the toxic effects of sterigmatocystin, just as they are to that of aflatoxin B_1 (Enomoto & Miyata, 1976). ICR white Swiss mice of both sexes fed a diet containing 5 ppm sterigmatocystin developed more pulmonary tumours than did the controls. However, the incidence of other tumours was not affected by the feeding of sterigmatocystin (Zwicker, Carlton & Tuite, 1974).

The present study was carried out to determine effects in mice of feeding 30 and 120 ppm sterigmatocystin in the diet.

EXPERIMENTAL

Test material. Sterigmatocystin was isolated from the mycelia of *Aspergillus versicolor* (Vuillemin) Tiraboschi and purified by the method of Hatsuda & Kuyama (1954). The purity of this compound (pale yellow needles; m.p. 244–245°C) was checked by means of chromatographic and spectroscopic methods.

Sterigmatocystin was mixed with the basal diet (CE-2 or CE-7, Clea Japan Ltd, Tokyo) at levels of 30

or 120 ppm before pelleting. CE-2 was used in the test diet for the first 22 wk of the treatment and CE-7 was used in the test diet for the remaining experimental period. Chemical analyses of pellets, carried out at the Division of Food Chemistry, National Institute of Hygienic Sciences, Tokyo by Mrs E. Isohata and Dr M. Uchiyama before and after the experiment, confirmed the required sterigmatocystin content.

Animals and experimental procedure. The strain BDF_1 female mice used in the study were hybrids, $C_{57}BL/6NCrj$ ♀ × DBA/2NCrj ♂, and were obtained from Charles River Japan, Inc., Atsugi, Kanagawa. The animals were kept in plastic cages, five to a cage, in an air-conditioned room and were given pelleted diet and tap water *ad lib.* The mice (5 wk old, mean initial weight 16·2 g) were divided into three groups and treated as described below.

Group 1 (controls). Fifty mice were fed the basal diet. Basal diet CE-2 in pellets was given for the first 22 wk of the treatment and basal diet CE-7 in pellets was fed for the remaining experimental period.

Group 2. Fifty-five female mice were given pelleted feed containing 30 ppm sterigmatocystin for 55 wk and then were given basal diet CE-7 in pellets containing no sterigmatocystin for the remainder of the experiment.

Group 3. Fifty-five mice were given a pelleted feed containing 120 ppm sterigmatocystin for 40 wk. Thereafter, they were given the control diet (CE-7 pellets) for 4 wk, followed by a further 11 wk on the diet containing 120 ppm sterigmatocystin. The surviving mice were fed CE-7 in pellets containing no sterigmatocystin for the remaining experimental period.

Five mice from each group were killed at wk 43 and ten from each group were killed at wk 68. All of the survivors were killed at wk 73. All of the animals were weighed weekly and food consumption was recorded. Those found dead or killed when moribund as well as those killed at the time intervals specified above were autopsied and all organs were examined macroscopically and were fixed in 10% buffered formalin. The livers, spleens and kidneys of each mouse as well as those organs that showed gross pathological changes were examined histologically. Sections of paraffin-embedded material were stained with haematoxylin and eosin and by additional special methods (silver impregnation, Masson's trichrome and periodic acid-Schiff stains) when necessary.

For electron microscopic examination, representative samples of the tumour tissues were fixed in 2·5% glutaraldehyde for 2 hr and then treated with 1% osmium tetraoxide buffered with sodium phosphate solution (0·1 M, pH 7·3) for 2 hr. The specimen blocks were dehydrated through a series of graded concentrations of ethanol and embedded in epoxy resin. Thin sections were cut with glass knives on a Porter-Bloom MT-2B ultramicrotome and stained with uranyl acetate and lead mixture solution. The sections were examined at 50 kv in a HS-7D electron microscope.

Liver changes were classified according to a classification of mouse liver lesions suggested by Frith & Ward (1980) and Frith, Baetcke, Nelson & Schieferstein (1980).

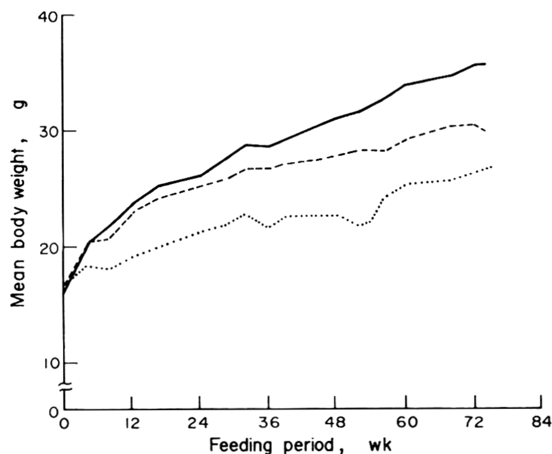

Fig. 1. Weight gain of mice fed 0 (——), 30 (---) or 120 (···) ppm sterigmatocystin in the diet. The group given 30 ppm sterigmatocystin was fed the test diet for 55 wk and then the control diet for the remainder of the experiment. The high-dose group was given the test diet for 40 wk then the control diet for 4 wk, followed by a further 11 wk on the test diet. Surviving mice were given the control diet for the remainder of the experiment.

RESULTS

The animals in the test groups gained weight more slowly than did the control group (Fig. 1). During the first 40 wk of the study the animals in the high-dose group, receiving a diet containing 120 ppm sterigmatocystin, weighed about 20% less than the control animals. This was probably due to reduced food intake and consequently after 40 wk the high-dose group was given control diet for 4 wk. Thereafter, the animals were given the test diet for a further 11 wk. Mice in both dose groups showed an increased weight-gain after wk 55, when sterigmatocystin treatment was discontinued. Calculated from the total consumption of diet, the average daily intake of sterigmatocystin was 97·2 μg/mouse in the low-dose group and 226 μg/mouse in the high-dose group during feeding of sterigmatocystin.

No animals in any of the three groups died before wk 43, except for one mouse in the high-dose group which died at wk 11. Histological study of this animal revealed atrophic changes in the thymus, liver and kidneys and karyorrhexis of germ centre cells in the lymph nodes and spleen.

No neoplastic changes were found in any of the mice killed at wk 43, and the only histological change observed was increased pleomorphism of liver cell nuclei in mice from the test groups (and especially from the high-dose group) in comparison with those from the control group.

Soft subcutaneous tumours, which showed fluctuating development, were observed on the backs of mice. Most of these were first observed about 50 wk after the start of the experiment. The tumours enlarged progressively and became firmer. Rupture of the tumour followed by death from haemorrhage was observed in one of the 21 mice of the low-dose group surviving 43–68 wk of the experiment and in 17 of 23 mice of the high-dose group surviving 61–68 wk of the experiment (Fig. 2).

Death following intra-abdominal bleeding was observed in 18 out of 21 mice in the low-dose group and one out of 23 mice in the high-dose group. Massive haemorrhage was caused by the rupture of vascular tumours that had developed in the liver, ovaries or lungs.

The only vascular changes observed in controls were peliosis-like lesions in the spleens of two mice.

Histological examination of tumours

The incidences of tumours in mice from the three groups are listed in Table 1. Two of the 50 animals in the control group developed tumours (one with a leiomyoma of the uterus and one with a leiomyosarcoma of the uterus). In the high- and low-dose groups tumours developed in 34 out of 55 and 44 out of 55 mice, respectively.

The subcutaneous tumours that developed on the backs of many of the mice were angiosarcomas. They were observed in six (12·5%) of the mice given 30 ppm sterigmatocystin and in 27 (58·7%) of those given 120 ppm. These vascular neoplasms which grew in a nodular form and ranged from 2 to 20 mm in diameter, were all found in the brown-fat tissue between the bilateral scapulas. Histologically, the tumours were composed of flattened, spindly or polygonal endothelial cells projecting into bloody spaces. Occasionally, multinucleated cells or cells with a giant hyperchromatic nucleus were found. Mitotic figures were frequently observed. Some areas within the tumours were packed with spindle-shaped or polygonal cells similar to those showing the vascular pattern characterized by cavernous spaces filled with blood (Fig. 3). Neither fatty vacoules nor glycogen-containing materials were demonstrated in these tumour cells by the use of specific stains. Reticulin-fibre stain showed the tumours to have a variety of structural patterns, such as double-framed cavities and interlacing basket-like frameworks (Fig. 4). The tumour cells infiltrated the neighbouring brown-fat tissue and frequently caused massive haemorrhage.

Hepatic haemangioendotheliomas and angiosarcomas occurred in a significantly greater number of mice in the low-dose group than in those of the high-dose group. Angiosarcomas of the liver were observed in 34 (64·2%) of the mice fed 30 ppm sterigmatocystin, but in none of those fed 120 ppm sterigmatocystin. Only four haemangioendotheliomas were found in the latter mice, compared with 14 in the low-dose group. Grossly, in those animals with vascular lesions, the liver exhibited multiple red cavities which ranged in size from minute spots to nodular masses. In the early

Table 1. *Incidence of tumours in female BDF_1 mice given control diet or diets containing sterigmatocystin*

			No. of mice with					
			Hepatic haem-	Angiosarcoma of the				
Sterigmatocystin dose (ppm in diet)*	Time of kill (wk)	No. of mice killed	angioendo-thelioma	Liver	Brown fat	Ovary	Lung	Other tumours
0	43	5	0	0	0	0	0	0
	58†	1	0	0	0	0	0	0
	68	10	0	0	0	0	0	0
	73	34	0	0	0	0	0	1 leiomyosarcoma of the uterus,
								1 leiomyoma of the uterus
Total...	—	50	0	0	0	0	0	2
30	43	5	0	0	0	0	0	0
	68	10	8	5	1	0	0	1 lung adenoma
								1 hepatocellular adenoma
								1 papilloma of the oral cavity
	43–68‡	23§	0	19	1	1	1	1 leiomyosarcoma of the uterus
	73	17	6	10	4	0	0	1 hepatocellular carcinoma
								1 leukaemia
								4 lung adenomas
Total...	—	55	14	34	6	1	1	10
120	11‖	1	0	0	0	0	0	0
	43	5	0	0	0	0	0	0
	68 0	10		0	2	1	0	3 lung adenomas
	61–68‡	27§	2	0	17	0	0	1 sarcoma of the uterus
								2 lung adenomas
								1 leiomyoma of the uterus
	73	12	2	0	8	2	0	1 squamous cell carcinoma of the auditory gland duct
								7 lung adenomas
								2 hepatocellular adenomas
Total...	—	55	4	0	27	3	0	17

*For details of feeding regimes see Experimental.
†Killed when moribund at time indicated.
‡Died or killed when moribund at time indicated.
§Two of these 23 mice in the 30-ppm group and four of these 27 in the 120-ppm group could not be examined because of cannibalism or autolysis.
‖Time of death.

stages of tumour development, hyperaemia and cystic dilation of sinusoids filled with blood were observed microscopically. These peliosis-like lesions were usually lined with proliferating endothelial cells and were considered to be haemangioendotheliomas. Severe compression of the liver-cell cords was not observed. However, in the later stage, enlarged blood-filled masses showed the irregularly dilated bloody spaces lined with highly anaplastic cells that suggested malignancy. Pleomorphic, hyperchromatic nuclei were marked in these flattened, spindle-shaped or polygonal cells (Fig. 5). Irregular invasion into the neighbouring hepatic parenchyma and a few cases of metastasis to the lungs were found.

Ovarian angiosarcomas occurred in one mouse in the low-dose group and in three of those in the high-dose group. One lung angiosarcoma occurred in the low-dose group. Metastases of angiosarcomas were rather exceptional. Almost all of the multiple angiosarcomas found in the same animal were probably primary tumours rather than tumours resulting from metastases.

Other types of tumours were also observed (Table 1). However, with the exception of lung adenomas and hepatocellular adenomas, none occurred frequently and in no case were they observed in mice in the test groups significantly more frequently than in the present controls or than in untreated mice of BDF_1 strain from the same breeder that have been observed previously by Tsubura (1981).

Histological examination of the liver

The incidence of histological changes other than vascular lesions in the liver are shown in Table 2. There was a dose-dependent increase in pleomorphism of liver cells. This pleomorphism was classified as cytomegaly and karyomegaly according to the criteria of Frith & Ward (1980). Proliferative hepatic lesions classified as foci of cellular alteration, hepatocellular adenoma and hepatocellular carcinoma were seen more frequently in the test groups than in the controls. However, no dose-dependent increase in the incidence of any of these lesions was found.

Electron microscopic examination of vascular tumours

Electron microscopically the hepatic angiosarcomas were composed of blood spaces lined by electron-lucent, flat or closely packed polygonal cells. Desmosomes and interdigitations were found between the tumour cells. Binocytotic vesicles were seen at the surface membranes. Occasionally, the cytoplasm projected deeply into the lumen. In general, the tumour cell had a cleaved nucleus with a prominant nuceolus. Mitotic figures were observed infrequently. The cytoplasm of the tumour cell was usually rich in rough endoplasmic reticulum which showed moderate dilation of the cisternae. Some of the tumour cells contained microtubular bodies like Weibel-Palade bodies (Shirai, 1981) in their cytoplasm.

The subcutaneous angiosarcomas were composed of flat or swollen, electron-lucent cells. These cells had a similar appearance to those of the hepatic angiosarcomas described above. In areas that consisted only of tumour tissue, the tumour cells and their nuclei had more angular or irregular shapes. The occasional presence of smooth muscle cells with characteristic myofibrils surrounding the basement membrane of the endothelial tumour cells (Fig. 6) also suggested the vascular origin of this tumour (Waldo, Vuletin & Kaye, 1977).

DISCUSSION

The present results clearly demonstrate that in BDF_1 female mice the long-term administration of sterigmatocystin in the diet resulted in the induction of malignant vascular tumours. Electron microscopy of hepatic and brown-fat tumours revealed typical vascular tumours composed of neoplastic endothelial cells. Seventy-five (72·1%) of the 104 sterigmatocystin-treated mice examined had one or more tumours of the blood vessels. The first tumour was found in the

Table 2. *Hepatocellular lesions observed in female BDF_1 mice given control diet or diets containing sterigmatocystin*

Sterigmatocystin dose (ppm in diet)*	Time of kill (wk)	No. of mice killed	No. of mice with hepatic lesions: Cytomegaly & karyomegaly: None	Slight	Moderate	Foci of cellular alteration: Acidophilic	Basophilic	Hepatocellular adenoma	Hepatocellular carcinoma
0	43	5	5	0	0	0	0	0	0
	68	10	0	9	1	0	0	0	0
	73	34	17	17	0	0	0	0	0
30	43	5	0	5	0	4	0	0	0
	68	10	0	5	5	3	6	1	0
	43–68†	21	2	9	10	7	4	0	0
	73	17	0	14	3	8	7	0	1
120	11‡	1	1	0	0	0	0	0	0
	43	5	0	2	3	4	0	0	0
	68	10	0	0	10	8	2	0	0
	61–68†	23	2	6	15	7	3	0	0
	73	12	0	4	8	3	1	2	0

*For details of feeding regimes see Experimental.
†Died or killed when moribund at time indicated.
‡Died at time indicated.

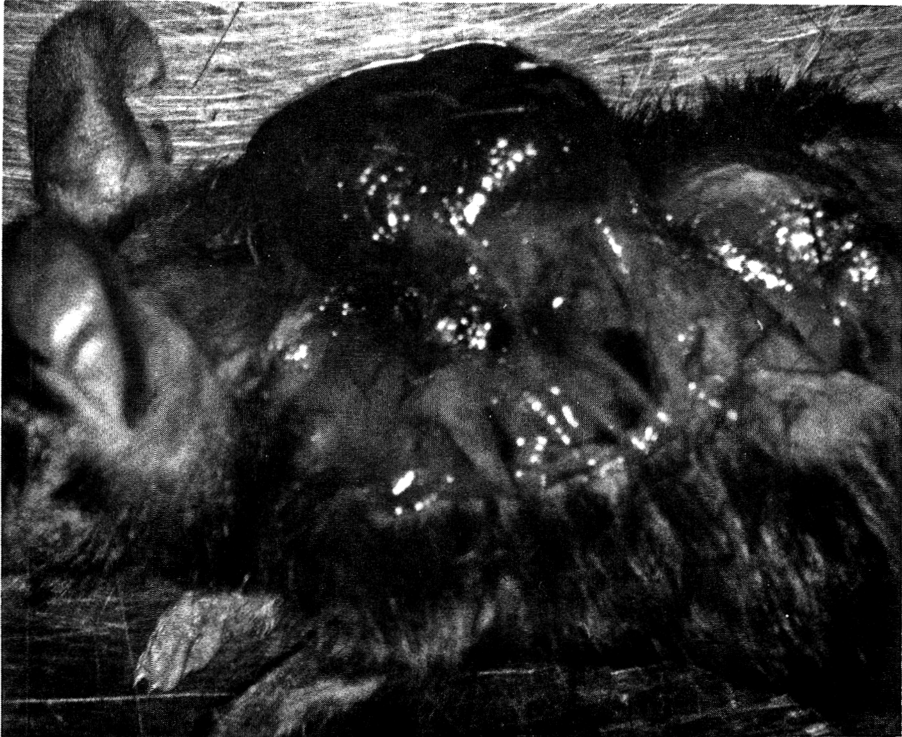

Fig. 2. A female mouse killed 68 wk after the beginning of the experiment. Angiosarcoma of the subcutis: the tumour growth occupies the brown-fat tissue on the back of the mouse.

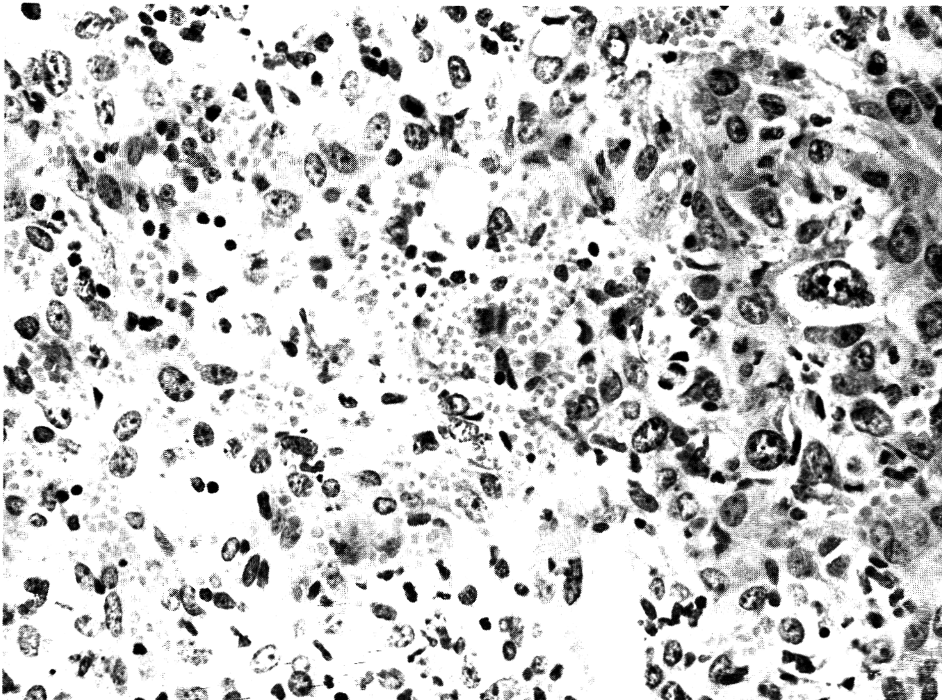

Fig. 3. Angiosarcoma of brown-fat tissue in a female mouse killed at wk 68. Note the vascular tumour growth with capillary and cavernous blood-filled spaces. Areas of closely packed polygonal tumour cells are also seen. Haematoxylin and eosin ×400.

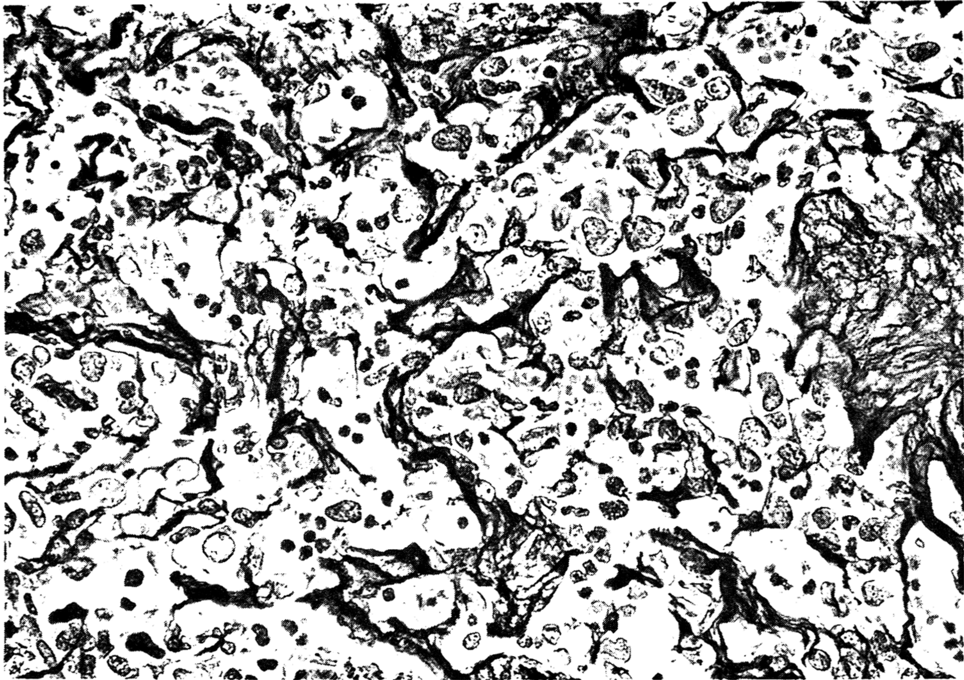

Fig. 4. Angiosarcoma of the brown-fat tissue in a female mouse killed at wk 73. Note the cavernous framework of the angiosarcoma and the fairly large nuclei of the endothelial tumour cells. Silver impregnation ×400.

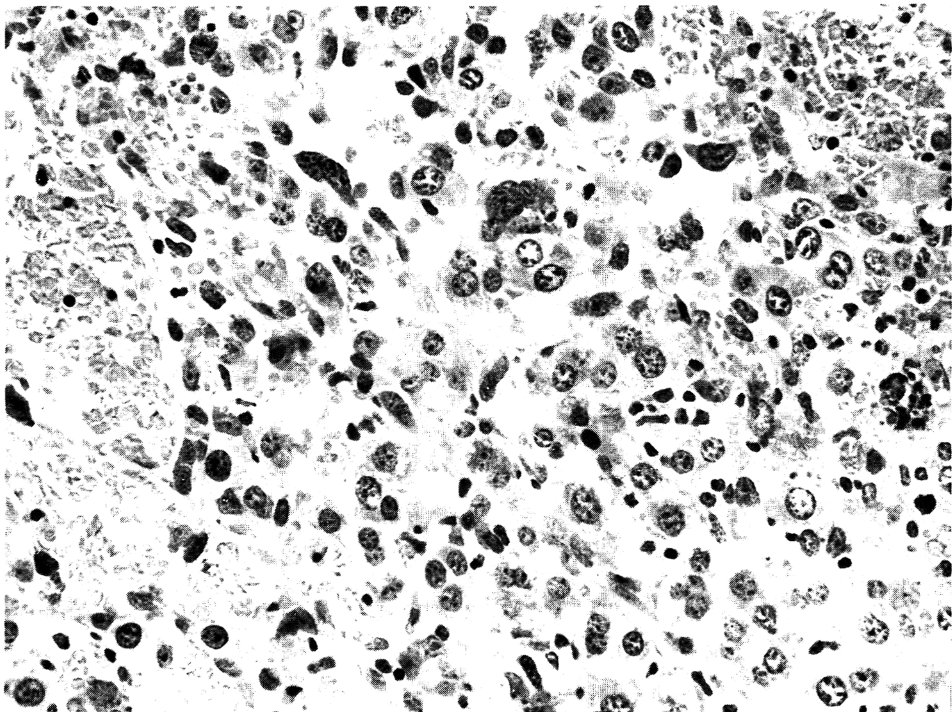

Fig. 5. Hepatic angiosarcoma in a female mouse killed at wk 68. Note the irregularly dilated bloody spaces lined by endothelial tumour cells which show nuclear pleomorphism. Liver cells are seen mixed with the tumour cells at the lower right-hand side of the picture. Haematoxylin and eosin ×400.

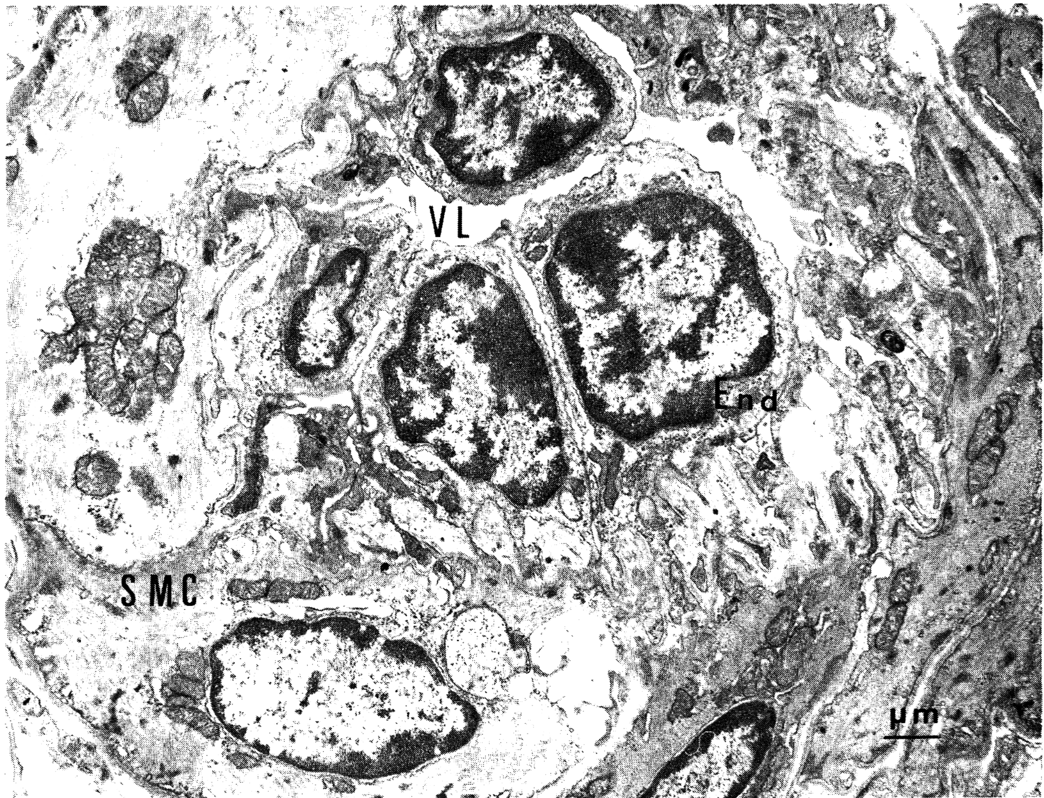

Fig. 6. Electron micrograph of an angiosarcoma in the brown-fat tissue of a female mouse killed at wk 68. Note a single layer of endothelial cells (End) and smooth muscle cells (SMC), irregular in shape and arrangement. × 10280.

brown-fat tissue on the back at wk 43. No vascular tumours occurred in 44 control mice observed for up to 73 wk. A recent study of spontaneous tumours in female BDF_1 mice, obtained from the same breeder, revealed no development of vascular tumours in untreated mice over a 2-yr period (Tsubura, 1981).

There are no reports available on the development of malignant vascular tumours in brown-fat tissue. Various incidences of blood vessel tumours have been induced in animals by carcinogenic chemicals, but although some of the vascular tumours induced by urethan (Deringer, 1962), *N*-nitrosodimethylamine (Toth, Magee & Shubik, 1964) and symmetrical dimethylhydrazine (Toth & Wilson, 1971) were found in fat other than pararenal tissue, there was only one case of a brown-fat tumour (in an HR/De mouse painted with urethane in Deringer's study). The brown fat, a type of adipose tissue commonly seen in young mammals and abundant even in adults in hibernating species, is known to be rich in vascular tissue. Its brown colour is thought to be derived from this vascular network and from the abundant lysosomes in the fat cells. It is interesting that in this study the target tissue for tumour induction by sterigmatocystin was the vascular elements of brown fat. In previous studies a low incidence of hepatic angiosarcomas has been induced in rats fed a diet containing sterigmatocystin for more than 1 yr (Mabuchi, 1980; Maekawa *et al.* 1979; Ohtsubo *et al.* 1978). One splenic haemangiosarcoma was seen in a rat treated with 1·5 mg sterigmatocystin/day for 42 wk (Purchase & van der Watt, 1970). Since the distribution and metabolic fate of sterigmatocystin in the animal body have been little investigated, it is not possible at present to give a clear interpretation of the toxic or carcinogenic effects of this mycotoxin on the target cells.

The other question of the significant differences in the sites of development of vascular tumours at the two dose levels of sterigmatocystin used in the present study also remains unresolved. The incidence of angiosarcomas in the brown-fat tissue was 11·3% in the low-dose group compared with 52·9% in the high-dose group. However, no angiosarcomas of the liver were induced in the high-dose group, whereas the incidence of hepatic angiosarcomas was high (64·2%) in the low-dose group. Benign haemangioendotheliomas were induced in the livers of four of the 51 mice examined in the high-dose group.

The liver is the usual target for the carcinogenic action of sterigmatocystin given orally to rats or medaka. In the present study only a few mice developed hepatocellular adenomas (one in the low-dose group and two in the high-dose group). One hepatocellular carcinoma was found in the low-dose group. Hepatocellular tumours were not observed in the control mice in our 74-wk study. The occurrence of such tumours was not reported in untreated female mice of the same strain observed for 2 yr (Tsubura, 1981). It is interesting that mice fed sterigmatocystin showed more cytomegaly and karyomegaly of the liver cells and foci of cellular alteration than did the control mice. Foci of hepatocellular alteration have been suggested to be possible precursors of hepatocellular tumours (Frith *et al.* 1980).

An interesting finding was an increase in the incidence of lung adenomas in test groups compared with the controls. Spontaneous lung adenomas were not observed in the controls. Tsubura (1981) reported an incidence of pulmonary tumours of 5·0% in males and 1·7% in females in untreated BDF_1 mice during a 2-yr period of observation. Zwicker *et al.* (1974) reported that both male and female mice of the ICR white Swiss strain fed a diet containing 5 ppm sterigmatocystin for 54–58 wk developed more pulmonary tumours than did the controls. They attributed this occurrence to a carcinogenic effect of sterigmatocystin.

Our previous study of the acute toxic effects of aflatoxin B_1 and sterigmatocystin in mice revealed relatively low susceptibility of this species to both mycotoxins. The single oral LD_{50}s were approximately 290 mg aflatoxin B_1/kg body weight and more than 800 mg sterigmatocystin/kg in DDD strain mice (Enomoto & Miyata, 1976). Mice are also known to be relatively insensitive to the carcinogenic effects of aflatoxin B_1 and sterigmatocystin. However, newborn mice treated with aflatoxin B_1 (Vesselinovitch, Mihailovich, Wogan *et al.* 1972) or sterigmatocystin (Fujii, Kurata, Odashima & Hatsuda, 1976) showed an increased susceptibility to the induction of hepatocellular tumours. In the present study, a relatively large amount of sterigmatocystin was given to both test groups of mice from 6 wk of age. Mice given 120 ppm sterigmatocystin in the diet consumed about 1·2% of the single oral LD_{50} or about 10 mg/kg body weight/day, a mean total intake of about 3·6 g/kg. Mice given 30 ppm sterigmatocystin consumed about 4 mg sterigmatocystin/kg body weight/day, a mean total intake of about 1·5 g/kg.

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BACTERIAL MUTAGENICITY STUDIES ON CHLOROFORM *IN VITRO*

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Abstract—Chloroform was tested for mutagenicity in the Salmonella/microsome assay using five strains of *Salmonella typhimurium*. In view of previous reports describing the development of liver and kidney tumours in some experiments involving long-term administration of chloroform to rats and mice, the mutagenicity tests were carried out in the absence of any S-9 microsomal-enzyme preparation and in the presence of S-9 microsomal-enzyme preparations derived from (a) livers and (b) kidneys of rats and mice previously exposed to the microsomal-enzyme inducer Aroclor 1254. No evidence of potential mutagenicity was observed under any of the test conditions. To determine whether the findings might have been influenced by the volatility of the chloroform, the test organisms were exposed to chloroform vapour, but again chloroform gave no indication of potential mutagenicity. Taken in conjunction with already published data from mutagenicity studies with chloroform, it appears unlikely that the tumours observed in some long-term rodent studies are attributable to a genotoxic action of the compound.

INTRODUCTION

In the first published account of tumorigenesis following repeated administration of chloroform to small rodents, Eschenbrenner & Miller (1945) noted that a dose level sufficient to produce liver necrosis seemed to be a prerequisite for hepatoma development. Ilett, Reid, Sipes & Krishna (1973) demonstrated covalent binding of chloroform metabolites to tissue proteins and associated this with the production of necrosis. These authors also showed how the kidney is at risk in circumstances in which the biotransformation of chloroform in the liver may be incomplete. Depletion of glutathione in the liver may be a determining factor in this toxicity, as indicated by Docks & Krishna (1976).

In recent long-term studies, chloroform administration has been associated with the development of liver tumours in B6C3F1 mice of both sexes and of kidney tumours in male Osborne-Mendel rats (National Cancer Institute, 1976); in other studies, kidney tumours have developed in male ICI mice (Roe, Palmer, Worden & Van Abbé, 1979) but not in females of this strain or males of three other strains, nor in male or female Sprague-Dawley rats (Palmer, Street, Roe *et al.* 1979) or beagle dogs (Heywood, Sortell, Noel *et al.* 1979). The lowest dose level at which tumour development has been reported in any species is 60 mg chloroform/kg/day; this produced tumours in the kidneys of male ICI mice, but no excess of tumours occurred when chloroform was given at 17 mg/kg/day to males or females of the same strain.

Although it is now apparent that most known chemical carcinogens increase the numbers of revertant colonies in one or more of the standard test strains of *Salmonella typhimurium* on histidine-deficient medium (Ames, McCann & Yamasaki, 1975), several workers (de Serres & Ashby, 1980; Simmon, Kauhanen & Tardiff, 1977; Uehleke, Werner, Greim & Krämer, 1977) have reported that chloroform does not give positive findings in this type of mutagenicity test. Such studies are usually conducted in the presence and absence of metabolizing enzymes provided by an S-9 microsome mix derived from the livers of rats that have been subjected to liver-enzyme induction by Aroclor 1254. In view of the findings in long-term rodent experiments, however, it seemed of interest to extend the range of bacterial mutagenicity testing to include the use of mouse-liver enzymes as well as rat-liver enzymes and also to examine the effect, if any, of microsomal enzymes derived from the kidneys of both species rather than from the liver. To avoid the possibility of 'false negative' results due to volatilization of the chloroform, an additional experiment was carried out in which chloroform vapour was caused to impinge continuously on the bacterial culture plates; prolonged chloroform exposure on these lines would eventually prove lethal to the organisms, but mutagenic activity, if any, might be detectable in the earlier stages of exposure to the vapour.

EXPERIMENTAL

Indicator organisms. Salmonella typhimurium strains TA1535, TA100, TA1537, TA1538 and TA98 were used as the histidine-dependent indicator organisms. Strain TA100 was obtained from ICI Pharmaceuticals Ltd, Macclesfield, Cheshire, and the other four from

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Professor B. N. Ames, University of California, Berkeley, CA, USA. The TA100 strain used was characterized as ampicillin-resistant despite the relatively low spontaneous level of revertants recorded. Stock suspensions in medium were stored frozen in liquid nitrogen and, before use, cultures prepared from the stocks were incubated in nutrient broth for 17 hr at 37°C; resulting suspensions contained approximately 2×10^9 organisms/ml and are referred to as the 'standard bacterial suspension'.

Culture media. Standard liquid medium for all strains was nutrient broth (Oxoid No. 2). The basal layer in plate cultures was Vogel-Bonner minimal agar medium (Difco poured plates); the upper layer, in which the bacteria were suspended, was a 0·6% solution of Difco bacto agar in 0·5% sodium chloride. Histidine-deficient medium was obtained by adding, to each 100 ml of this agar solution, 10 ml of a solution of 0·5 mM-histidine hydrochloride and 0·5 mM-biotin. For histidine-rich medium, 10 ml of a solution of 0·1 M-histidine hydrochloride and 50 mM-biotin was added to each 100 ml of agar solution.

Chemicals. Chloroform (pharmaceutical grade) was obtained from ICI Pharmaceuticals Ltd. The known mutagens used as positive controls were β-naphthylamine and 2-aminoanthracene (from Sigma London Chemical Co., Kingston, Surrey), neutral red (from Raymond A. Lamb Ltd, London) and 2-acetylaminofluorene (from Aldrich Chemical Co., Milwaukee, WI, USA), all of which require oxidation by microsomal enzymes to produce mutagenic metabolites, and sodium azide (from Sigma London Chemical Co.) and 4-nitro-*o*-phenylenediamine (from Aldrich Chemical Co.), which do not require metabolic activation. All chemicals were dissolved in dimethylsulphoxide (DMSO), obtained from Sigma London Chemical Co.

Microsomal-enzyme fraction. Liver S-9 fractions were prepared from specific-pathogen-free rats of the CFY (Sprague-Dawley-derived) strain and mice of the ICI/CFLP strain. The animals were 8–10 wk old and were obtained from Anglia Laboratory Animals, Alconbury, Cambridgeshire. For the kidney S-9 fractions, specific-pathogen-free CD (Sprague-Dawley-derived) rats and CD1 (ICR-derived) mice were obtained from Charles River (UK) Ltd, Margate, Kent. Following acclimatization for 1 wk, each animal was injected ip with Aroclor 1254 (200 mg/ml) dissolved in arachis oil, at a dose level of 500 mg/kg body weight, to stimulate microsomal-enzyme activity. Five days after injection, and following a 16-hr period of fasting, the animals were killed by cervical dislocation. Under sterile conditions, the kidneys or livers (as appropriate) were removed and minced in 0·15 M-KCl (5 g tissue in 15 ml KCl) before transfer to a Potter homogenizer (Measuring and Scientific Equipment Ltd, Loughborough, Leics), for homogenization at 4°C. Pooled homogenates were centrifuged at 9000 g for 15 min in a refrigerated centrifuge (MSE 25). The supernatant fraction ('S-9') was stored in 3-ml lots at −20°C for not more than 4 wk and was thawed immediately before use for the preparation of S-9 mix following the procedure of Ames *et al.* (1975), 0·3 ml S-9 per ml S-9 mix being used.

Test procedures

Preliminary toxicity test. The same procedure was used for each strain. Minimal agar (15 ml) was poured into 90-mm Petri dishes and allowed to gel. The standard bacterial suspension (0·1 ml) was added to 3 ml histidine-deficient agar at 45°C and the resulting suspension was overlaid on the minimal agar gel. When the upper layer had set, wells 10 mm in diameter were cut in the gel and into these were pipetted 0·1-ml aliquots of a series of DMSO solutions containing 0–10 mg chloroform/ml. Dishes were incubated for 24 hr at 37°C, after which time bacterial toxicity was assessed by measuring the zones of inhibition around each well.

Mutation study (standard procedure). For each indicator strain, 0·1-ml aliquots of the chloroform dilutions were pipetted into bijou bottles, three sets—each of three bottles—being used for each dilution. Similar sets received 0·1-ml aliquots of DMSO or solutions of graded concentrations of the positive control compounds. The standard bacterial suspension (0·1 ml) was added to each bottle. Into each bottle of one of the sets was dispensed 0·5 ml of the rat S-9 mix. This was replaced in the second set by 0·5 ml of the mouse S-9 mix, and in the third by 0·5 ml of sterile 0·9% sodium chloride. The complete series was duplicated for the kidney mixes.

To each bottle, 2·8 ml of histidine-deficient agar was added at 45°C and after thorough mixing the resulting suspension was overlaid on to a previously prepared gel of 15 ml of minimal agar in a 90-mm Petri dish. Triplicate dishes were used to test each concentration of each chemical. After incubation at 37°C for 48 hr, revertant bacterial colonies on each plate were counted with an electronic colony counter (Biotran Mk II, New Brunswick Scientific Co., Inc., Edison, NJ, USA).

Vapour phase study. The procedure differed from the standard technique insofar as the bacterial inoculum and (where appropriate) the microsomal extract (rat-liver S-9 mix) were spread over the surface but the co-factors were incorporated into the top agar. The plates were then placed in an anaerobic jar at 37°C and a stream of chloroform vapour was passed through the jar using a Millipore miniature vacuum/pressure pump (type XX6 122050; Millipore (UK) Ltd, London) to give a flow rate of 0·03 m^3/min. The vapour stream gave a mean passage of 32 ml chloroform/hr in triplicate runs. 'Negative' control plates were treated with a stream of air for comparable periods. After the required exposure time, all plates were taken from the jars and further incubated to give a total 72-hr incubation. As positive controls, ethyl methanesulphonate (2·5%) and 2-acetylaminofluorene (50 μg/plate) were used (applied on discs, not as vapour).

RESULTS

In the preliminary toxicity test, chloroform was apparently non-toxic to all five *S. typhimurium* strains at a concentration of 10,000 μg/well. However, in the bacterial mutagenicity study (Table 1) a concentration of 10,000 μg/plate was toxic for all strains, as shown by the formation of an incomplete bacterial lawn. At and below 1000 μg chloroform/plate the bacterial lawns were satisfactory but there was no significant increase in revertant colonies for any of the indicator

Table 1. *Mutagenicity testing of chloroform in five strains of* Salmonella typhimurium *with/without microsomal-enzyme preparations (standard procedure)*

S. typhimurium strain	Dose of $CHCl_3$ (μg/plate)	No. of revertants/plate*					
		Without activation	With liver S-9 mix		Without activation	With kidney S-9 mix	
			From rat	From mouse		From rat	From mouse
TA1535	0	10	14	12	13	13	10
	10	7	14	12	12	13	16
	100	9	13	13	11	10	15
	1000	7	12	12	14	12	10
	10,000	IL	IL	IL	IL	IL	IL
TA1537	0	6	4	7	7	14	9
	10	2	5	5	7	12	7
	100	3	6	3	9	11	10
	1000	4	5	2	6	11	10
	10,000	IL	IL	IL	IL	IL	IL
TA1538	0	9	14	12	12	20	18
	10	9	12	15	13	22	18
	100	9	14	15	12	18	19
	1000	7	14	14	12	20	15
	10,000	IL	IL	IL	IL	IL	IL
TA98	0	32	45	53	31	30	31
	10	36	42	36	32	30	33
	100	40	45	39	29	33	30
	1000	27	43	45	34	36	30
	10,000	IL	IL	IL	IL	IL	IL
TA100	0	22	28	26	63	70	62
	10	13	22	28	67	74	69
	100	13	25	22	64	67	71
	1000	17	21	25	70	75	69
	10,000	IL	IL	IL	IL	IL	IL

IL = Incomplete bacterial lawn
*Values are means for triplicate plates. Positive-control results are presented in Table 2.

strains, either in the absence of metabolic activators or in the presence of microsomal enzymes from rat or mouse S-9 mix derived from liver or kidney. By contrast, the known mutagens (positive controls) caused large increases in the mutation frequency of the five bacterial strains under these experimental conditions in the presence or absence of metabolic activation, as appropriate (Table 2).

In the vapour phase study, the passage of chloroform vapour for 6–8 hr was highly toxic to strains TA1535 and TA1538. No significant increase in revertant colonies could be detected at any earlier stage,

Table 2. *Mutagenicity of positive-control compounds in five strains of* Salmonella typhimurium *with microsomal-enzyme activation where appropriate (standard procedure)*

S. typhimurium strain	Compound	Dose (μg/plate)	No. of revertants/plate*				
			Without activation	With liver S-9 mix		With kidney S-9 mix	
				From rat	From mouse	From rat	From mouse
TA1535	Sodium azide	5	977	—	—	—	—
	β-Naphthylamine	10	—	136	169	—	—
	2-Aminoanthracene	2	—	—	—	246	151
TA1537	4-NPDA	500	136	—	—	—	—
	Neutral red	10	—	51	51	55	51
TA1538	4-NPDA	500	1559	—	—	—	—
	2-AAF	20	—	106	210	242	174
TA98	4-NPDA	500	1865	—	—	—	—
	2-Aminoanthracene	2	—	318	369	1717	151
TA100	Sodium azide	5	1128	—	—	—	—
	2-Aminoanthracene	2	—	245	333	1146	429

— = Not tested 4-NPDA = 4-Nitro-*o*-phenylenediamine 2-AAF = 2-Acetylaminofluorene
*Values are means for triplicate plates.

Table 3. *Vapour-phase tests for mutagenicity of chloroform in* Salmonella typhimurium *strains TA1535 and TA1538 with/without rat-liver microsomal-enzyme preparations*

Treatment	No. of revertants/plate*			
	TA1535		TA1538	
	Without S-9 mix	With S-9 mix	Without S-9 mix	With S-9 mix
None	22	26	33	45
EMS (2·5%)	c. 200	—	c. 200	—
2-AAF (50 μg/plate)	—	c. 500	—	c. 500
$CHCl_3$ vapour—2 hr	27	24	31	42
4 hr	13	27	0	40
6 hr	5	27	0	2
8 hr	0	0	0	3

— = Not tested EMS = Ethyl methanesulphonate 2-AAF = 2-Acetylaminofluorene
*Values are means for triplicate plates.

either in the absence of metabolic activation or in the presence of rat liver S-9 mix (Table 3).

DISCUSSION

The distinction between the apparent non-toxicity of chloroform at 10,000 μg/well in the preliminary toxicity test and the incomplete bacterial lawn noted in the mutagenicity experiments at 10,000 μg chloroform/plate may have been related to volatilization of chloroform from the wells in the preliminary test. Growth of an incomplete lawn at the high dose level in the mutagenicity experiments, however, shows that under these conditions sufficient chloroform remained to exert an antibacterial effect. Table 3 shows that the findings for mutagenicity are identical even when care is taken to make sure that contact with chloroform is not impaired through volatilization. Hence it appears that the mutagenicity findings given in Table 1 correctly reflect the lack of mutagenic activity attributable to chloroform under the various conditions of testing. Whilst confirming the negative findings already reported by other authors, it can now be seen that chloroform is not mutagenic to the five standard indicator strains of *S. typhimurium* in the presence of metabolizing enzymes from either the kidney or the liver, whether obtained from rats or mice.

These findings seem to be fully consistent with those of Diaz Gomez & Castro (1980), who were unable to detect covalent binding of chloroform metabolites to liver DNA or RNA of male Sprague–Dawley rats or of male A/J strain mice after multiple chloroform administration. The development of tumours in small rodents in the course of long-term studies involving repeated intragastric administration of chloroform is therefore unlikely to be a consequence of any direct genotoxic action of the compound. Relevant to the search for an alternative mechanism may be the conclusion reached much earlier by Eschenbrenner & Miller (1945) that chloroform needed to be given at a dose-level sufficient to cause necrosis of the liver in order to provoke the formation of liver tumours in mice.

Agustin & Lim-Sylianco (1978) recently reported that in mutagenicity tests against *S. typhimurium* TA1537 positive findings were obtained with urine concentrates from male mice treated with chloroform at a dose level of 700 mg/kg. At and above a similar dose level, these authors also obtained positive results in a micronucleus test, but dose levels of chloroform up to 400 mg/kg were inactive. Although these findings suggest that mutagenic metabolites are formed when chloroform is given to male mice at very high dose levels, they probably do not account for the enhanced renal-tumour risk encountered in earlier studies in response to chloroform at dose levels of only 60 mg/kg/day (Roe *et al.* 1979).

The mechanism by which chloroform may lead to the development of tumours in the liver or kidneys of mice or rats, when given repeatedly at dose levels insufficient for detectable mutagenic effect, remains to be elucidated. The dose levels at which excess tumours developed when chloroform was given repeatedly to rodents greatly exceeded the dose levels to which humans are exposed when they use products incorporating chloroform as a preservative or flavouring agent. It seems likely that a non-genotoxic mechanism was involved under the experimental conditions that gave rise to these tumours.

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EVALUATION OF THE CUTANEOUS-IRRITATION POTENTIAL OF 56 COMPOUNDS

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Abstract—The primary cutaneous irritation of 56 chemicals was tested in the rabbit using three different procedures. The three protocols selected for the tests were the method published by the French authorities for the testing of cosmetics and toiletries (Journal Officiel 21 April 1973, p. 3862; *ibid* 5 June 1973, p. 3953) and the methods proposed for the testing of chemicals by the Association Française de Normalisation (AFNOR) and, in 1979, by the Organisation for Economic Co-operation and Development. The results of the three sets of tests were compared and the effects of differences in procedure and numbers of animals were studied, together with the possible relation between irritancy and the pH of the test material. It was concluded that the AFNOR protocol best met the requirements for such tests and that gloves should be worn for the handling of all substances classified as moderately or severely irritant on that scale.

INTRODUCTION

Various protocols have been developed for determining in the rabbit the local effects of chemicals on the skin. In a comparative study carried out at IFT, 56 substances have been tested by three of the available methods. Those selected were the method published by the French authorities for the testing of cosmetics and toiletries (Journal Officiel de la République Française (1971 & 1973) and those proposed by AFNOR (Association Française de Normalisation, 1982) and by the OECD (Organisation for Economic Co-operation and Development, 1979), identified in this text as the Cosmetic, AFNOR and OECD protocols, respectively. The study was designed to establish the validity of these protocols and to examine the possibilities of making practical recommendations on the handling of the test substances.

EXPERIMENTAL

Protocols. The principles of the three protocols chosen and the differences between them are outlined in Table 1. To facilitate the interpretation and comparison of the results, certain minor procedural differences between the methods were eliminated by the use of a standardized procedure for preparing the test substances, preparing the skin and applying the test substances.

Animals. The tests were carried out on male albino rabbits of the New Zealand strain, weighing about 2·5 kg and supplied by Roucher SA, Couhé. Each test material was tested by the three (occlusive) protocols in a single group of six rabbits, so that comparison of the results was not confused by variations in individual reactions. The exception to this arrangement was the use of a separate group of six rabbits for each protocol for one test compound (no. 8, dimethyl sulphate), the percutaneous toxicity of which following the triple application was sufficient to kill several of the animals. A fresh goup of six rabbits was also used for each substance tested by the semi-occlusive OECD protocol.

Test materials. Liquids, semi-liquids and powders were used without pretreatment; other solids were pulverized prior to application. Powdered test substances were not moistened, as specified by the OECD protocol, but were applied dry and the gauze patch covering the powder was moistened with 0·5 ml water. Where possible, the pH of the substance, of its saturated aqueous solution or of the organic phase of its 50/50 (v/v) aqueous solution was measured with a Tacussel pH meter.

Skin preparation. Each rabbit was clipped over the back and flanks with a fine-toothed electric clipper (Aesculap Type V 42 947), to give a precise cut (height 0·05 mm) without causing mechanical irritation of the

Abbreviations: AFNOR = Association Française de Normalisation: PCI = primary cutaneous irritation index.

Table 1. *Test protocols for the evaluation of cutaneous irritation and/or corrosivity in the rabbit*

Protocol*		
Cosmetic	**AFNOR**	**OECD**
	Animal species and number	
Six male albino rabbits of about 2·5 kg	Six male albino rabbits of about 2·5 kg	At least three adult albino rabbits (recommended species)
	Preparation of animals	
Clipping	Clipping	Clipping or chemical depilation
	Preparation of test substance	
Liquids or semi-liquids: no particular preparation	Liquids or semi-liquids: no particular preparation	Liquids or semi-liquids: no particular preparation
Powders or solids (that can be pulverized): mode of prepration chosen by the Study Director†	Powders or solids (that can be pulverized): mode of preparation chosen by the Study Director†	Powders or solids (that can be pulverized): moistening of the test substance
	No test on strongly alkaline (pH $\geqslant$ 11·5) or acidic (pH $<$ 2) substances	No test on strongly alkaline or acidic substances
	Application of the test substance	
Exposure to 0·5 ml or g of test substance for 23 hr with and without scarification on an area of approx. 4 cm^2. Use of an occlusive dressing	Exposure to 0·5 ml or g of test substance for 4 hr with and without scarification on an area of approx. 4 cm^2. Use of an occlusive dressing	Exposure to 0·5 ml or g of test substance for 4 hr without scarification on an area of approx. 6 cm^2. Use of a semi-occlusive dressing
	Longer exposure possible according to intended use of test substance	Longer exposure possible according to intended use of test substance, and repetition of the test with occlusive dressing if necessary
	Observation times	
At 1 and 48 hr after patch removal	At 1, 24 and 48 hr after patch removal Possible extension of observation period to 7 days and up to 14 days to evaluate (ir)reversibility of lesions	At 30–60 min, and then at 24, 48 and 72 hr after patch removal Possible extension of observation period up to 14 days to evaluate (ir)reversibility of lesions
	Expression of results	
Calculation of primary cutaneous irritation index from the numerical evaluation of erythema and oedema formation	Calculation of primary cutaneous irritation index from the tabulated evaluation of erythema and oedema formation	No determination of cutaneous irritation index but tabulated evaluation of erythema and oedema formation
		Description of effects recorded at each observation period
	Interpretation of results	
Interpretation of the index according to published scale (*Journal Officiel* 21.4.71)	Interpretation of the index according to an evaluation scale proposed by ETAD (Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry)	Results interpreted by the Study Director

*Cosmetic—official method for testing cosmetics and toiletries (*Journal Officiel de la République Française*, 1971 & 1973); AFNOR—proposed by Association Française de Normalisation (1982) for testing chemicals; OECD—also proposed for testing chemicals (OECD, 1979).
†Gauze patch moistened with 0·5 ml water.

skin. The animals were left for 24 hr and then only those with healthy and glabrous skin were selected for the tests. The location of all the treatment sites on the dorsal region of the trunk ensured that all materials were applied to skin of identical histological structure. For the comparison of the three occlusive protocols, the clipped surface was divided into three parts, care being taken to leave sufficient space between the test areas to prevent interference between the applications. In both the anterior (Cosmetic protocol) area and the median (AFNOR) area, the right flank was scarified by three superficial parallel incisions, 20 cm long and 0·5 cm apart, using a sterile vaccination lancet. The incisions were restricted to the epidermis; if the dermis was damaged and bleeding occurred the animal was discarded. The posterior part of the clipped area was used for the OECD (occlusive) test and the clipped skin was left intact on both flanks.

Application of test substances

Occlusive tests. For the application of liquids, two sterile four-layered hydrophilic-gauze pads (2 × 2 cm in area) were placed on the skin of each rabbit in each of the three test areas, one pad on the right flank (scarified for the Cosmetic and AFNOR protocols) and one on the left (intact) side. A dose of 0·5 ml of the test liquid was then applied to each gauze pad by means of a sterile 2·5-ml polypropylene syringe (from Becton Dickinson (UK) Ltd, Wembley, England). Semi-liquids in doses of 0·5 ml and pastes and powders in doses of 0·5 g (weighed on a Mettler balance, type PL 200 [d = 1 mg] and kept in a heparin-free haemolysis tube) were applied directly to the right and left flanks of the same three areas (scarified or intact as appropriate) and the treated skin was covered in each case with a four-layered sterile absorbent-gauze square (2 × 2 cm), which was moistened with 0·5 ml water if the test substance was a powder. The test material and gauze pads were kept in contact with the skin by a patch (Neodermotest Roc SA, Paris) consisting of an occlusive central disc (22 mm in diameter) surrounded by an adhesive hypoallergenic perforated plaster (10 mm wide). Finally an adhesive tape (6 cm wide) was wound round the animal, without restricting respiratory and abdominal movements, to complete the holding of the patches, and the rabbit was placed in a restraining device for at least 4 hr.

OECD semi-occlusive protocol. The same doses of test substances were applied as for the occlusive protocols, but the absorbent gauze pads (again moistened with 0·5 ml water for the application of powders) were approximately 6 cm^2 in area instead of 4 cm^2 as above. No patch was added, but the pads were held in place with adhesive tape, as before.

Readings and interpretation of results

Readings. The pads were removed after 23 hr (for the Cosmetic protocol) or after 4 hr (AFNOR and both OECD procedures). Macroscopic observations were recorded at 1 and 48 hr after patch removal for the Cosmetic protocol, at 1, 24 and 48 hr for the AFNOR tests and at 1, 24, 48 and 72 hr for both types of OECD test. Further readings at day 7 and day 14 were added to the AFNOR and OECD observations in cases of pronounced irritation, to evaluate the possible reversibility of the lesions.

Histological examinations. Where the colour of a test substance made the macroscopic examination of erythema impossible, histological examination of cutaneous biopsies, stained with haematein–eosin, was undertaken (AFNOR, 1982). Since such examinations could not distinguish between scarifications and irritant lesions, scarification of the right flanks was omitted in these cases, so the AFNOR and OECD tests differed only in the timing of the biopsies, which were taken at the same intervals as the reading times given above, up to a maximum of 72 hr.

Scoring. Each treated area was scored for erythema and oedema using a numerical system (0–4 in each case according to severity) based on that described by Draize, Woodard & Calvery (1948). For any one of the protocols, the scores obtained for erythema and oedema at both of the treated sites in all six animals at the two or more reading times were totalled. The sum obtained was then divided by the total number of readings to provide a mean score (never greater than 8) termed the *primary cutaneous irritation index* (PCI). For this purpose, the total numbers of readings (erythema + oedema) for the Cosmetic, AFNOR and OECD protocols were 24, 36 and 48, respectively, corresponding to two application sites on six animals at 1 and 48 hr, at 1, 24 and 48 hr, and at 1, 24, 48 and 72 hr, respectively, after removal of the patches.

Interpretation. The irritancy of each test substance was then defined on the basis of the PCI, using the scale appropriate for each protocol. The Cosmetic protocol provides for classification of irritancy on the following scale: PCI below 0·5, non-irritant; 0·5–2, slightly irritant; 2–5, moderately irritant; 5–8, severely irritant. This scale differs in one respect from that published earlier (Journal Officiel de la République Française, 1971), which classified as slightly irritant all compounds with a PCI above 0 and below 2. Numerous products in the pure undiluted state have a PCI below 2 and the newer classification of a substance as non-irritant if its PCI is below 0·5 enables a distinction to be made between a very slight reaction of unknown cause and a clearly visible irritation caused by the product. The slightly different AFNOR scale (PCI below 0·5, non-irritant; 0·5–3, slightly irritant; 3–5, moderately irritant; 5–8, severely irritant) was used also to interpret the results of the OECD tests, since no scale is identified in the OECD protocol. It should be noted that the latter protocol defines *cutaneous irritation* as the production of reversible inflammatory changes in the skin and *cutaneous corrosion* as the production of irreversible tissue damage in the skin, following application of a test substance. This aspect was investigated where appropriate by readings on days 7 and 14 after treatment.

RESULTS

The 56 substances tested and the results obtained using the three (or sometimes four) procedures described are listed in Table 2.

Non-irritant test substances

Thirty of the test substances were classified as non-irritant on the basis of all three occlusive tests. These

Table 2. *Identification of 56 test substances and their primary cutaneous irritation indices determined in the rabbit by the Cosmetic, AFNOR and OECD protocols*

Serial no.	Test material	Appearance	Approx. pH*	Test procedure†	PCI‡	Classification
1	Mineral oil, sterile codex	Colourless viscous liquid	—	Cosmetic	0·08	NI
				AFNOR	0·08	NI
				OECD	0·04	NI
2	Anhydrous lanolin, USP	Thick yellow emulsion	—	Cosmetic	0·17	NI
				AFNOR	0·11	NI
				OECD	0·06	NI
3	Propylene glycol, USP	Colourless liquid	8·8 (a)	Cosmetic	0·17	NI
				AFNOR	0·14	NI
				OECD	0·02	NI
4	Toluene	Colourless liquid	—	Cosmetic	3·25	MI
				AFNOR	3·42	MI
				OECD	2·94	SlI
				OECD semi-occlusive	2·13	SlI
5	Ethanol, 90°	Colourless liquid	8·3 (a)	Cosmetic	0·21	NI
				AFNOR	0·03	NI
				OECD	0	NI
6	White spirit, dilutine 5	Colourless liquid	—	Cosmetic	2·21	MI
				AFNOR	2·56	SlI
				OECD	2·58	SlI
				OECD semi-occlusive	1·92	SlI
7	Scouring powder, Ajax lemon	Beige powder	10·7 (b)	Cosmetic	1·42	SlI
				AFNOR	0·06	NI
				OECD	0·02	NI
8	Dimethyl sulphate	Colourless liquid	1·0 (c)	Cosmetic§	7·88	SvI
				AFNOR§	7·08	SvI
				OECD§	7·60	SvI
				OECD semi-occlusive	5·98	SvI
9	4-Dimethylsulphamido-2-amino-2′-nitrodiphenyl sulphide	Yellow powder	9·6 (b)	Cosmetic	0·21	NI
				AFNOR	0·14	NI
				OECD	0	NI
10	4-Dimethylsulphamido-2-formamido-2′-nitrodiphenyl sulphide	Yellow powder	5·3 (b)	Cosmetic	0·13	NI
				AFNOR	0·22	NI
				OECD	0·08	NI
11	3-Dimethylsulphamidophenothiazine	Browny-yellow powder	9·9 (b)	Cosmetic	0·33	NI
				AFNOR	0·25	NI
				OECD	0·17	NI
12	3-Cyanophenothiazine (pure)	Yellow powder	6·4 (b)	Cosmetic	0	NI
				AFNOR	0	NI
				OECD	0	NI
13	Cyamepromazine	Yellow powder	8·2 (b)	Cosmetic	0	NI
				AFNOR	0·05	NI
				OECD	0	NI

14	Promethazine	White solid lump (pulverized for PCI)
15	10-(3′-Dimethylamino-2′-methyl-1′-propyl)-3-methoxyphenothiazine (RS)	White powder
16	Oxomemazine	White powder
17	Iminodibenzyl	Beige powder
18	Chloropropylmethylpiperazine (20%, w/w, in toluene)	Colourless liquid
19	Tetraaminopyrimidine (sulphate)	Yellow powder
20	4-Chloro-5-sulphamoyl-phthalimide	Brown powder
21	Metformine (hydrochloride)	Beige powder
22	Tetraethylthiuram disulphide	White powder
23	1-Dimethylaminopropan-2-ol	Colourless liquid
24	1-Chloro-2-methyl-3-dimethylaminopropane (50%, w/w, in toluene)	Colourless liquid
25	Dipotassium ethylenediaminetetraacetate	White powder
26	Potassium lactate (purified, 60% aqueous solution)	Colourless liquid
27	Sodium lactate (purified, 60% aqueous solution)	Colourless liquid
28	*N*-(4-Methylamino)benzoylglutamic acid, sodium salt	Brown granules (pulverized for PCI)

8·7 (b)	Cosmetic	0·42	NI
	AFNOR	0·14	NI
	OECD	0·06	NI
8·3 (b)	Cosmetic	0	NI
	AFNOR	0	NI
	OECD	0	NI
9·0 (b)	Cosmetic	0	NI
	AFNOR	0	NI
	OECD	0	NI
7·5 (b)	Cosmetic	0	NI
	AFNOR	0·08	NI
	OECD	0	NI
10·7 (c)	Cosmetic	3·58	MI
	AFNOR	3·33	MI
	OECD	3·75	MI
	OECD semi-occlusive	1·98	SlI
2·5 (b)	Cosmetic	0·04	NI
	AFNOR	0·08	NI
	OECD	0·06	NI
3·6 (b)	Cosmetic	1·71	SlI
	AFNOR	1·14	SlI
	OECD	0·04	NI
7·6 (b)	Cosmetic	1·38	SlI
	AFNOR	0·89	SlI
	OECD	0·13	NI
5·1 (b)	Cosmetic	0	NI
	AFNOR	0·03	NI
	OECD	0	NI
10·5 (a)	Cosmetic	5·33	SvI
	AFNOR	4·05	MI
	OECD	4·42	MI
	OECD semi-occlusive	4·23	MI
8·4 (a)	Cosmetic	4·33	MI
	AFNOR	4·11	MI
	OECD	4·21	MI
	OECD semi-occlusive	1·90	SlI
5·2 (b)	Cosmetic	0·79	SlI
	AFNOR	1·14	SlI
	OECD	0·40	NI
8·1 (a)	Cosmetic	0·17	NI
	AFNOR	0·39	NI
	OECD	0	NI
8·0 (a)	Cosmetic	0·08	NI
	AFNOR	0·28	NI
	OECD	0·04	NI
7·6 (b)	Cosmetic	0·38	NI
	AFNOR	0·78	SlI
	OECD	0·29	NI

[*contd*]

Table 2—c

Serial no.	Test material	Appearance
29	*p*-Iodobenzoyl glutamic acid (pure)	Beige granules (pulverized for PCI)
30	Dibenzoyltartaric acid	White powder
31	Embonic acid (technical)	Yellow powder
32	2,4-Dichloro-5-sulphamoylbenzoic acid	Beige powder
33	2,4-Dichloro-5-chlorosulphonylbenzoic acid	Beige powder
34	Ammonium isethionate	Small white crystals
35	1,6-Dibromohexane	Colourless liquid
36	Caprylylcystinic acid	White powder
37	1-Palmitoyl-4-palmitoyloxyproline	Beige powder
38	Palmitoylcystinic acid	White powder
39	*o*-Aminophenol	Beige crystals (pulverized for PCI)
40	*p*-Aminophenol	White powder
41	Sodium fluosilicate	White powder

continued

Approx. pH*	Test procedure†	PCI‡	Classification
3·9 (b)	Cosmetic	0·08	NI
	AFNOR	0·05	NI
	OECD	0·02	NI
2·6 (b)	Cosmetic	0	NI
	AFNOR	0	NI
	OECD	0·02	NI
6·2 (b)	Cosmetic	0·33	NI
	AFNOR	0·36	NI
	OECD	0·25	NI
2·7 (b)	Cosmetic	0·25	NI
	AFNOR	0·06	NI
	OECD	0	NI
2·1 (b)	Cosmetic	1·08	SII
	AFNOR	0·39	NI
	OECD	0·19	NI
5·2 (b)	Cosmetic	0·21	NI
	AFNOR	0·31	NI
	OECD	0·04	NI
5·2 (a)	Cosmetic	1·96	SII
	AFNOR	1·92	SII
	OECD	1·63	SII
3·2 (b)	Cosmetic	0·83	SII
	AFNOR	0·06	NI
	OECD	0·02	NI
4·7 (b)	Cosmetic	1·17	SII
	AFNOR	0·06	NI
	OECD	0·06	NI
4·4 (b)	Cosmetic	0·04	NI
	AFNOR	0	NI
	OECD	0	NI
7·1 (b)	Cosmetic	0	NI
	AFNOR	0	NI
	OECD	0	NI
7·3 (b)	Cosmetic	0·21	NI
	AFNOR	0·03	NI
	OECD	0	NI
3·5 (b)	Cosmetic	1·63	SII
	AFNOR	0·86	SII
	OECD	0·04	NI

42	Copper nitrate	Blue crystals (pulverized for PCI)
43	Ammonium ferrioxalate	Green crystals (pulverized for PCI)
44	Aluminium nitrate	White crystals (pulverized for PCI)
45	Oxalic acid	White powder
46	Cyclopentanone	Colourless liquid
47	*o*-Diethoxybenzene	Colourless liquid
48	Iminophenacyl thiazolidine bromhydrate	Light beige powder
49	*p*-Phenetidine	Light brown liquid
50	Orthovanilline	Yellow chips (pulverized for PCI)
51	Mepyramine	Beige powder
52	Reticulable silica paste in polysiloxane	Greyish paste
53	Polysiloxane resin	Light yellow viscous liquid
54	Polysiloxane resin (30% w/w, in toluene)	Colourless viscous liquid

1·2 (b)	Cosmetic		H (A)‖
	AFNOR		H (A)‖
	OECD		H (A)‖
	OECD semi-occlusive		H (A)‖
5·7 (b)	Cosmetic	4·22	MI
	AFNOR	3·06	MI
	OECD	0·30	NI
0·8 (b)	Cosmetic	0·54	SlI
	AFNOR	0·36	NI
	OECD	0·35	NI
1·0 (b)	Cosmetic	4·50	MI
	AFNOR	4·03	MI
	OECD	1·44	SlI
	OECD semi-occlusive	0·92	SlI
5·0 (a)	Cosmetic	2·21	MI
	AFNOR	2·75	SlI
	OECD	3·00	SlI
	OECD semi-occlusive	0·31	NI
—	Cosmetic	0·42	NI
	AFNOR	0·17	NI
	OECD	0·21	NI
3·5 (b)	Cosmetic	0·17	NI
	AFNOR	0·14	NI
	OECD	0	NI
10·8 (a)	Cosmetic	0·58	SlI
	AFNOR	0·67	SlI
	OECD	0·04	NI
4·2 (b)	Cosmetic		H (B)‖
	AFNOR		H (B)‖
	OECD		H (B)‖
	OECD semi-occlusive		H (C)‖
9·2 (b)	Cosmetic	0·04	NI
	AFNOR	0·06	NI
	OECD	0·02	NI
5·6 (b)	Cosmetic	2·04	MI
	AFNOR	1·78	SlI
	OECD	1·27	SlI
	OECD semi-occlusive	0·73	SlI
4–5 (a)	Cosmetic	0·33	NI
	AFNOR	0·17	NI
	OECD	0·04	NI
7–7·5 (a)	Cosmetic	1·88	SlI
	AFNOR	2·69	SlI
	OECD	2·77	SlI
	OECD semi-occlusive	2·35	SlI

[*contd*]

Table 2—*continued*

Serial no.	Test material	Appearance	Approx. pH*	Test procedure†	PCI‡	Classification
55	Silica paste in polysiloxane	Pasty emulsion	10·1 (a)	Cosmetic	0·25	NI
				AFNOR	0·11	NI
				OECD	0·08	NI
56	γ-Glycidoxypropyltrimethoxysilane	Colourless liquid	6·9 (a)	Cosmetic	1·54	SlI
				AFNOR	1·75	SlI
				OECD	0·21	NI

PCI = Primary cutaneous irritation index NI = Non-irritant SlI = Slightly irritant MI = Moderately irritant
SvI = Severely irritant H = Histological examination

*pH of (a) named substance, (b) its saturated aqueous solution or (c) the organic phase of a 50/50 (v/v) aqueous solution.
†For details of test procedures, see Experimental section. Except where specified otherwise, the OECD results were obtained using an occlusive dressing.
‡PCI = total score obtained (erythema + oedema)/no. of readings. The numbers of readings were 24 for the Cosmetic protocol, 36 for the AFNOR protocol and 48 for the OECD protocols.
§Separate groups of rabbits were used for each protocol because of the high percutaneous toxicity of this compound.
‖Histological examination (H) of biopsies of skin exposed to compound 42 or 50 showed (A) a severely irritant reaction, up to 72 hr in the case of the OECD semi-occlusive test, (B) irritant reaction with eschar formation or (C) no pathological effects at 1, 24, 48 or 72 hr.

substances were mineral oil (no. 1), anhydrous lanolin (no. 2), propylene glycol (no. 3) and ethanol (no. 5), together with nos 9–17, 19, 22, 26, 27, 29–32, 34, 38–40, 47, 48, 51, 53 and 55.

Slightly irritant test substances

The substances considered under this heading are those for which at least one of the three protocols gave a slightly irritant (but no higher) classification. The variations in classification could be ascribed in some cases to the varying severity of the tests, in some to the different observation times and in others to differences in the interpretation scales.

Only two substances (nos 35 and 54) were classed as slightly irritant by all three protocols, five (nos 7, 33, 36, 37 and 44) were slightly irritant by the Cosmetic protocol and non-irritant by AFNOR and OECD, and six (nos 20, 21, 25, 41, 49 and 56) were deemed slightly irritant by the Cosmetic and AFNOR protocols and non-irritant by OECD. No. 28 was classified as slightly irritant by the AFNOR protocol (PCI 0·78) and as non-irritant by the Cosmetic (PCI 0·38) and OECD protocols.

For four other slightly irritant compounds (nos 25, 49, 54 and 56), the AFNOR-derived PCI was higher than the Cosmetic but the difference (which was very small for nos 49 and 56) was not reflected in a change in classification. This was due in the case of no. 54 (Cosmetic PCI 1·88; AFNOR PCI 2·69) to the less 'severe' classification scale provided by the AFNOR and OECD protocols, in which the top limit for the slightly irritant class is a PCI of 3, as opposed to 2 in the Cosmetic protocol. The AFNOR result on compound no. 54 was confirmed in the OECD tests with PCI values of 2·77 (occlusive test) and 2·35 (semi-occlusive).

Several samples (nos 20, 21, 25, 28, 41, 49 and 56), classed as slightly irritant by the AFNOR method (involving applications with and without scarification), were non-irritant according to the OECD, which did not include application to scarified tissue.

Reversibility. With slightly irritant substances with a PCI below 2, the lesions were reversible by day 7 and, in most cases, by 48 or 72 hr after removal of the patches. With compound no. 54 the only test substance in this group with a PCI above 2 (AFNOR and OECD), the lesions were not reversed by day 14 in the AFNOR and OECD occlusive tests, although they were reversed by day 7 in the semi-occlusive OECD test. Taking into account the slow reversibility of the lesions would put this compound in the moderately irritant category.

Moderately to severely irritant test substances

Substances given these classifications according to at least one protocol included three (nos 6, 46 and 52) with a Cosmetic PCI between 2 and 3 and consequently a classification of moderate irritancy. Seven others were classified as moderately or severely irritant, having a PCI above 3 by at least one protocol. For four of these ten substances (nos 6, 23, 46 and 52), the irritancy classification was one category higher by the Cosmetic protocol than by AFNOR or OECD, while for no. 43, the Cosmetic PCI (4·22) was markedly higher than the AFNOR value (3·06), although the classification was the same in both cases. In three

cases (nos 4, 43 and 45) the AFNOR classification was moderately irritant, compared with slightly irritant or non-irritant by the OECD protocol.

Histological examinations. The colour of copper nitrate (no. 42) and of orthovanilline (no. 50) prevented a macroscopic assessment of erythema (and therefore calculation of the PCI). Histological examination of biopsy samples of skin treated under occlusion with these compounds showed a markedly irritant (orthoergic) reaction. With semi-occlusive treatment, no. 42 showed the same effect, but no pathological reaction was seen with no. 50.

Results with OECD occlusive and semi-occlusive protocols. In addition to nos 42 and 50, ten other materials were tested by the OECD semi-occlusive method as well as by the OECD occlusive protocol. For seven (nos 4, 6, 8, 23, 45, 52 and 54) both methods yielded the same classification, but compared with the occlusive test, the semi-occlusive test resulted in a classification one category lower with nos 18, 24 and 46.

Reversibility. The lesions caused by the compounds (nos 4, 46 and 52) with a PCI of less than 3 were generally reversible by day 14. However, when the PCI was above 3 the lesions showed only slight regression by day 14, when erythema was still clearly visible and oedema was occasionally recorded.

Influence of group size

To assess the advantage, if any, of using six rabbits, the results from each group of six rabbits were randomly divided into two sets of three and the calculations were repeated first on one set and then on the other. For 13 materials (nos 4, 6, 14, 23, 26, 28, 31, 35, 43, 44, 46, 52 and 53) the classification deduced from one set of three animals differed in at least one protocol from that obtained from the whole group. These differences occurred most frequently with the Cosmetic protocol.

DISCUSSION

For 37 of the 56 test materials, all three occlusive protocols resulted in the same irritancy classification. In other cases, differences involved only neighbouring categories, except with ammonium ferrioxalate (no. 43), which was classified as moderately irritant by the Cosmetic and AFNOR protocols but as non-irritant by the OECD.

Influence of protocol variations

The results of this study indicate that the AFNOR protocol, specifying application of the test substance to scarified and intact skin for only 4 hr under occlusion, provides in most cases a fairly reliable evaluation of irritation. When applied for 23 hr under occlusion (Cosmetic protocol), only nine samples caused a more severe irritation resulting in a higher classification than that obtained with the 4-hr exposure (Table 3). Moreover, the 23-hr application seems the less relevant to normal conditions of exposure.

The few cases in which the shorter (AFNOR) application to scarified and intact skin resulted in a higher PCI (leading, in one case only, to a higher irritancy classification) may have been due to the absence of a 24-hr observation in the Cosmetic protocol and partial reversal of the lesions by the 48-hr reading.

The calculations carried out on the results of randomly divided groups of three rabbits suggest that reducing the group size from six to three would reduce the reliability of these tests.

Influence of pH

Comparison of the pH of the test materials with the results obtained showed that strongly acid substances were not necessarily corrosive. Thus while dimethyl sulphate (no. 8; pH 1·0) was severely irritant and copper nitrate (no. 42, pH 1·2) caused a marked histologically detectable reaction, oxalic acid (no. 45; pH 1·0) was classified as only moderately irritant and aluminium nitrate (no. 44; pH 0·8) as non-irritant (AFNOR protocol in each case). It seems unnecessary, therefore, to restrict all testing for primary cutaneous irritancy to substances with a pH above 2 (as specified for example in the AFNOR protocol). Results on samples with a pH between 2 and 3 supported the lack of a consistent association between low pH and severe irritancy, samples no. 19 (pH 2·5), no. 30 (pH 2·6), no. 32 (pH 2·7) and no. 33 (pH 2·1) all being in the non-irritant (AFNOR) category. However, of these eight materials only dimethyl sulphate was a liquid, the others being solids.

Similarly varying results were obtained with sub-

Table 3. *Differences in irritancy classification following testing by the Cosmetic and AFNOR protocols*

Test substance		Grade of irritancy	
No.	Identity	Cosmetic protocol	AFNOR protocol
7	Scouring powder	SlI	NI
33	2,4-Dichloro-5-chlorosulphonylbenzoic acid	SlI	NI
36	Caprylylcystinic acid	SlI	NI
37	1-Palmitoyl-4-palmitoyloxyproline	SlI	NI
44	Aluminium nitrate	SlI	NI
6	White spirit	MI	SlI
46	Cyclopentanone	MI	SlI
52	Reticulable silica paste in polysiloxane	MI	SlI
23	1,1-Dimethylaminopropan-2-ol	SvI	MI
28	*N*-(4-Methylamino)benzoylglutamic acid, sodium salt	NI	SlI

NI = non-irritant SlI = Slightly irritant MI = Moderately irritant SvI = Severely irritant

stances of high pH, but in these cases the physical state was perhaps less relevant. Thus according to the AFNOR protocol, the one solid giving a pH measurement above 10 (scouring powder, no. 7, pH 10·7) was non-irritant, three liquids, nos 18 (pH 10·7), 23 (pH 10·5) and 49 (pH 10·8) were moderately, moderately and slightly irritant, respectively, and a fourth (no. 55, a pasty emulsion of pH 10·1) was non-irritant.

Recommendations on handling precautions

We consider that appropriate protective measures should be compulsory for the handling of any substances determined by the AFNOR protocol to have a PCI above 3. Among the substances tested in this study, the wearing of gloves is recommended for the handling of the liquids numbered 4, 8, 18, 23 and 24, and the solids numbered 43 and 45, as well as for two other solids (nos 42 and 50) shown by histological examination to induce irritant reactions.

When tested by the OECD protocol, cyclopentanone (no. 46) provoked lesions that were not always reversible by day 14, although the PCI calculated was equal to 3, giving a classification of slightly irritant. On the other hand, when this substance was tested by the AFNOR protocol, the lesions were almost completely reversed by day 14 and a PCI of 2·75 again gave a slightly irritant classification. In such a case, in which a border-line effect is observed, it would seem prudent for handling precautions to be taken.

However, in most cases where the AFNOR and OECD protocols yielded different classifications, the latter method gave the less severe result. Thus in contrast to the conclusions drawn on the basis of the AFNOR protocol, special handling precautions would not be indicated by the OECD results on toluene (no. 4, PCI (occlusive) 2·94), ammonium ferrioxalate (no. 43, PCI (occlusive) 0·30) and oxalic acid (no. 45, PCI (occlusive) 1·44 (semi-occlusive) 0·92), the PCI being below 3 in each case. Moreover, the semi-occlusive test, which is specified in the OECD protocol as the first test to be used, always gave a lower PCI than was deduced from the occlusive test, which together with the possible extension of the application time in accordance with conditions of use, is merely offered as a possible additional test. The results of these comparative studies demonstrated, therefore, that the OECD protocol is insufficiently severe. Experience has shown, for example, that it is advisable for gloves to be worn when toluene is being used.

Conclusions

Because some of the instructions in the Cosmetic and/or OECD protocols are imprecise, it was necessary to make a selection regarding procedures for fur removal, the application of dry substances and the interpretation of results. Our experience has shown that clipping is preferable to chemical depilation, as the latter is itself likely to irritate the skin and interfere with results. Direct moistening of powders or other solids on application is considered inadvisable, since it may increase skin penetration and create a situation different from normal conditions of use. Loss of the substance during the experiment is prevented by covering. Regarding interpretation of results, calculation of the PCI does not, in itself, seem to serve a useful purpose; it is preferable to define a scale by which the index can be used to deduce the grade of irritancy.

This comparative study showed that it was necessary to use groups of six rabbits in order to obtain reliable results and good reproducibility and demonstrated that a 4-hr occlusive application on scarified and intact skin was an adequate exposure for determining irritancy. Moreover this procedure reflected the most severe conditions likely to be encountered in use, when a workman with scratched hands could be exposed to a compound encrusted under his gloves. It also became evident that for irritant compounds it was necessary to determine the reversibility of the lesions.

We consider that these criteria are best met by the AFNOR protocol, which has the added advantages of being sufficiently precise to give a good prospect of interlaboratory agreement and of relating more closely than the other protocols to practical conditions of exposure. Use of this protocol should help manufacturers to recommend appropriate handling precautions to users of their products.

Since the results of these protocols depend on the subjective reading of macroscopic reactions (Weil & Scala, 1971), controls are essential to ensure good reproducibility and reliability. In the IFT laboratory, technicians who are solely responsible for applying test substances and reading the reactions always conduct the examinations 'blindly' (i.e. without knowledge of the scores obtained at previous readings). To ensure that all the technicians are reading the lesions in the same way, a monthly check is made in which they all examine the same group of animals and compare their results.

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EVALUATION OF THE OCULAR-IRRITATION POTENTIAL OF 56 COMPOUNDS

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Abstract—The ocular irritancy of 56 chemicals was tested in the rabbit eye, with and without rinsing, using a procedure that complied with the main requirements of three protocols, namely those proposed by the Association Française de Normalisation (AFNOR) in 1982 and by the Organisation for Economic Co-operation and Development in 1979 for the testing of chemicals and that published by the French authorities (*Journal Officiel* 21 April 1971, p. 3862; *ibid* 5 June 1973, p. 3953) for the testing of cosmetics and toiletries. The results obtained were used to establish the extent to which the assessment of a chemical's irritation potential was affected by the differences between the three protocols in respect of the observation times prescribed, the inclusion and timing of rinsing, the number of animals used and the interpretation procedure. Conclusions were also drawn about the influence of the physical state and pH of a material on its potential irritancy and about the level of irritancy that necessitates the use of eye protection.

INTRODUCTION

Various protocols have been established for determining the local effects of chemicals on the rabbit eye. This paper describes the results obtained on 56 substances tested at IFT in a manner designed to permit a comparison between three of the available methods. Those selected were the official method approved by the French authorities for the testing of cosmetics and toiletries (*Journal Officiel de la République Française*, 1971 & 1973), and the methods proposed by AFNOR (Association Française de Normalisation, 1982) and by the OECD (Organisation for Economic Co-operation and Development, 1979). These three procedures are identified in this text as the Cosmetic, AFNOR and OECD protocols, respectively. The aim was to establish the validity of these protocols and to make practical recommendations on the handling of the test substances.

EXPERIMENTAL

Experimental procedure

Protocols. The principles of the three protocols studied and the essential differences between them are summarized in Table 1. To facilitate their comparison, a standardized procedure was used for all the substances, as indicated below, and the observations recorded were then interpreted in accordance with the various recommendations.

Animals. Male albino rabbits of the New Zealand strain, weighing about 2·5 kg and obtained from Roucher SA, Couhé, were tested in groups of six.

Test materials. Liquids and semi-liquids required no preparation. Solid test substances were ground, if necessary, to produce a fine dust for instillation. This involved a deviation from the Cosmetic protocol, which specifies 20% (w/v) aqueous dilution of powders. Where possible, the pH of the test substance, of its saturated aqueous solution or of the organic phase of its 50/50 (v/v) aqueous solution was determined.

Rinsing solution. Rinsing, when required, was generally carried out with Dacryoserum (from Chibret Laboratories, Paris), a solution of boric acid (1·8 g), sodium borate (1·2 g), sodium chloride (0·3 g) and phenylmercury borate (0·002 g) in 100 ml rose distilled water QSP. An alternative, generally that recommended by the manufacturer, was used when the use of Dacryoserum was contra-indicated by the available information on the nature of the test material.

Dosing and observation. Test substances were instilled into the lower conjunctival cul-de-sac of one eye of each rabbit; the other eye constituted the con-

Abbreviations: AFNOR = Association Française de Normalisation; AOI = acute ocular irritation index; IOI = individual ocular irritation index; MOI = mean ocular irritation index.

Table 1. *Test protocols for the evaluation of primary ocular irritation and/or corrosivity in the rabbit*

Protocol*		
Cosmetic	AFNOR	OECD
	Animal species and number	
Six male Albino rabbits of about 2·5 kg	Six male Albino rabbits of about 2·5 kg	At least 3 adult Albino rabbits
	Preparation of test substance	
Liquids or semi-liquids: no particular preparation Powders: 20% (w/v) dilution in sterile distilled water	Liquids or semi-liquids: no particular preparation Powders or solids (that can be pulverized): type of preparation chosen by the Study Director No test on strongly alkaline (pH ⩾11·5) or acidic (pH <2) substances	Liquids, semi-liquids or powders: no particular preparation Solid or granular material: ground to a fine dust No test on strongly alkaline (pH ⩾11·5) or acidic (pH <2) substances
	Application of test substance	
Dose 0·1 ml or 100 mg	Dose 0·1 ml or 100 mg If very irritant substance, test repeated in 6 additional rabbits, with rinsing of the eyeball and related structures 60 sec after instillation	Dose 0·1 ml or 100 mg If very irritant substance, test repeated in 6 additional rabbits, with rinsing of the eyeball and related structures with water in 3 rabbits 4 sec after the instillation, and in the other 3 rabbits 30 sec after instillation
	Observation times	
At 1, 2, 3, 4 and 7 days after instillation	At 1 hr and 1, 2, 3, 4 and 7 days after instillation. Possible extension of observation period to 14 or 21 days to evaluate (ir)reversibility of lesions	At 1, 24 and 72 hr after instillation Extended observation if the test article is irritant
	Expression of results	
No determination of ocular irritation index but tabulated evaluation of lesions of the cornea, iris and conjunctiva	Calculation of acute, mean and individual ocular irritation indices from the tabulated evaluation of the lesions of the conjunctiva, iris and cornea	No determination of ocular irritation index but tabulated evaluation of the lesions of the cornea, iris and conjunctiva Description of reactions observed at each observation period
	Interpretation of results	
Results interpreted by the Study Director	Indices interpreted according to an evaluation scale adapted from Kay & Calandra (1962), which considers both the severity of the reaction at a given time and the development of the lesions in the course of time	Results interpreted by the Study Director

*Cosmetic—official method for testing cosmetics and toiletries (*Journal Officiel de la République Française*, 1971 & 1973); AFNOR—proposed by Association Française de Normalisation (1982) for testing chemicals; OECD—also proposed for testing chemicals (OECD, 1979).

Table 2. *AFNOR scale for interpretation of ocular irritation evaluations*

Values of indices*			
AOI	MOI	Day-7 IOI	Conclusion
0–5	0 after 48 hr		Non-irritant (NI)
5–15	< 5 after 48 hr		Slightly irritant (SlI)
15–30	< 5 after 4 days		Irritant (I)
30–60	≤ 20 after 7 days	≤ 30 in all 6 rabbits	
		≤ 15 in at least 4/6	Very irritant (VI)
60–80	≤ 40 after 7 days	≤ 60 in all 6 rabbits	
		≤ 30 in at least 4/6	Severely irritant (SvI)
80–110†			Extremely irritant (EI)

*If the requirements for the value of an index are not met, the conclusion from the line below is chosen.
†Theoretical maximum score, from total of 80 (cornea) + 10 (iris) + 20 (conjunctiva).

trol. For liquids and semi-liquids a dose of 0·1 ml was instilled by means of a 1-ml sterile polypropylene syringe (from Becton Dickinson (UK) Ltd, Wembley, England). Pastes, powders and solids (ground if necessary) were instilled in a dose of 100 mg, weighed on a Mettler balance, type PL 200 (d = 1 mg), the powders being kept in a heparin-free haemolysis tube. Observations were recorded at 1 hr and 1, 2, 3, 4 and 7 days after instillation and, in cases where lesions persisted, also at later stages.

Test materials showing any degree of irritancy more severe than that classified as slightly irritant (see below) in this first test were similarly instilled into another group of six rabbits, and the eyeball and related structures were rinsed 30 sec after the instillation. If the material was still found to be irritant, a third test was run with rinsing after 4 sec. Approximately 20 ml of the rinsing solution, at about 20°C, was applied to the test eye and to the control, using a plastic wash-bottle. Excess liquid was removed with sterile gauze.

Scoring and classification of findings

Since the Cosmetic and OECD protocols specify no numerical system for the scoring and interpretation of results, a scoring system based on the AFNOR recommendations was used throughout, with modifications where appropriate to enable direct comparisons between the protocols to be made.

At each observation time, lesions of the conjunctiva, iris and cornea were scored separately using a numerical system based on that of Kay & Calandra (1962), the sum of these scores being the *individual ocular irritation index* (IOI) for each animal. Calculation of the mean (and standard deviation) of the IOI values obtained at any one time gave the *mean ocular irritation index* (MOI). The greatest MOI obtained over the range of observation times was identified as the *acute ocular irritation index* (AOI), which was used for the preliminary classification of a substance in one of six categories between 'non-irritant' and 'extremely irritant' (Table 2). In the AFNOR interpretation, which takes into account not only the maximum intensity of the lesion but also its reversibility, the preliminary classification is modified if recovery is not virtually complete within 4 days, by evaluating the fall in MOI with time and, after 7 days, the frequency of the persisting lesions (Table 2, columns 2 and 3, respectively). The classification resulting from the use of this procedure was therefore compared with that obtained directly from the AOI (corresponding to the OECD approach).

RESULTS

The list of substances tested, their appearance and pH and the degree of irritation demonstrated are presented in Table 3, together with the irritancy classification derived in accordance with the AFNOR protocol. By this method, 18 of the 56 test substances were found to be non-irritant or slightly irritant, 23 irritant or very irritant and 15 severely or extremely irritant when applied without subsequent rinsing.

Non-irritant and slightly irritant test substances

In this group, anhydrous lanolin (no. 2) was designated non-irritant following instillation of 0·1 ml without subsequent rinsing. Eleven materials—nos 1 (mineral oil), 3 (propylene glycol), 10, 12, 17, 27, 38, 40, 51, 53 and 56—were slightly irritant but caused no corneal opacity. However, slight corneal opacity was seen 1 hr and sometimes 24 hr after treatment in some rabbits treated with one of the other six slightly irritant substances (nos 9, 11, 22, 26, 34 and 39). All the lesions generally disappeared within 2 days of the instillation, and because of this reversibility and the absence of corrosion, tests with rinsing were not carried out.

Irritant and very irritant test substances

Tests without rinsing. In the irritant/very irritant group, the 14 substances designated irritant included toluene (no. 4), white spirit (no. 6) and dibromo-1,6-hexane (no. 35). The two latter solvents caused no detectable corneal opacity. White spirit and silica paste in polysiloxane (no. 55) were classed as irritants because the MOI exceeded 5 after 48 hr, but the AOI (i.e. the maximum MOI) for each of these materials was below 15. The nine very irritant substances included scouring powder (no. 7) and a 30% (w/w) solution of polysiloxane resin in toluene (no. 54). The latter, with an AOI of 35·67, is notable because toluene (AOI 22·67) was classed as irritant and polysiloxane resin alone (no. 53) as only slightly irritant (AOI 7·50). While the maximum MOI for most test compounds classed as irritant or very irritant was

Table 3. *Identification of 56 test substances and the ocular irritation indices determined in the rabbit in accordance with the principles of the proposed AFNOR protocol*

Serial no.	Test material	Appearance	Approx. pH*	Rinsing procedure†	AOI‡	MOI‡	IOI‡	Classification
1	Mineral oil, sterile codex	Colourless viscous liquid	—	None	10·83	0 [2]	—	SII
2	Anhydrous lanolin, USP	Thick yellow emulsion	—	None	2·67	0 [2]	—	NI
3	Propylene glycol, USP	Colourless liquid	8·8 (a)	None	11·33	0·83 [2]	—	SII
4	Toluene	Colourless liquid	—	None	22·67	4·83 [4]	—	I
				30 sec	13·33	2·33 [2]	—	SII
5	Ethanol, 90°	Colourless liquid	8·3 (a)	None	47·33	32·83 [7]	—	SvI
				30 sec	35·83	13·17 [7]	< 15 (5)	VI
				4 sec	37·50	6·50 [7]	< 15 (6)	VI
6	White spirit, dilutine 5	Colourless liquid	—	None	14·67	9·83 [2]	—	I
				30 sec	4·00	1·33 [2]	—	SII
7	Scouring powder, Ajax lemon	Beige powder	10·7 (b)	None	36·33	1·50 [7]	< 15 (6)	VI
				30 sec	21·33	0·67 [4]	—	I
				4 sec	17·33	0 [4]	—	I
8	Dimethyl sulphate	Colourless liquid	1·0 (c)	None	109·00§	—	—	EI
					13·00			
				30 sec	106·67	94·33 [7]	—	EI
					43·33	32·67 [7]		
				4 sec	100·17	—	—	EI
					53·50			
9	4-Dimethylsulphamido-2-amino-2′-nitrodiphenyl sulphide	Yellow powder	9·6 (b)	None	9·67	0 [2]	—	SII
10	4-Dimethylsulphamido-2-formamido-2′-nitro-diphenyl sulphide	Yellow powder	5·3 (b)	None	6·50	0 [2]	—	SII
11	3-Dimethylsulphamidophenothiazine	Browny-yellow powder	9·9 (b)	None	8·17	0 [2]	—	SII
12	3-Cyanophenothiazine (pure)	Yellow powder	6·4 (b)	None	8·17	0 [2]	—	SII
13	Cyamepromazine	Yellow powder	8·2 (b)	None	19·17	0·33 [4]	—	I
				30 sec	16·33	0 [4]	—	I
				4 sec	4·50	0 [2]	—	NI
14	Promethazine	White solid lump (pulverized for test)	8·7 (b)	None	61·00	35·67 [7]	> 30 (4)	EI
				30 sec	29·00	12·17 [4]	—	VI
				4 sec	25·17	4·33 [4]	—	I
15	10-(3′-Dimethylamino-2′-methyl-1′-propyl)-3-methoxyphenothiazine (RS)	White powder	8·3 (b)	None	16·17	0 [4]	—	I
				30 sec	14·67	0 [2]		SII
16	Oxomemazine	White powder	9·0 (b)	None	28·33	1·50 [4]	—	I
				30 sec	17·83	0 [4]	—	I
				4 sec	6·83	0·83 [2]	—	SII
17	Iminodibenzyl	Beige powder	7·5 (b)	None	9·17	0·67 [2]	—	SII

18	Chloropropylmethylpiperazine (20%, w/w, in toluene)	Colourless liquid	10·7 (c)
19	Tetraaminopyrimidine (sulphate)	Yellow powder	2·5 (b)
20	4-Chloro-5-sulphamoylphthalimide	Brown powder	3·6 (b)
21	Metformin (hydrochloride)	Beige powder	7·6 (b)
22	Tetraethylthiuram disulphide	White powder	5·1 (b)
23	1,1-Dimethylaminopropan-2-ol	Colourless liquid	10·5 (a)
24	1-Chloro-2-methyl-3-dimethylaminopropane (50%, w/w, in toluene)	Colourless liquid	8·4 (a)
25	Dipotassium ethylenediaminetetraacetate	White powder	5·2 (b)
26	Potassium lactate (purified, 60% aqueous solution)	Colourless liquid	8·1 (a)
27	Sodium lactate (purified, 60% aqueous solution)	Colourless liquid	8·0 (a)
28	*N*-(4-Methylamino) benzoyl glutamic acid, sodium salt	Brown granules (pulverized for test)	7·6 (b)
29	*p*-Iodobenzoyglutamic acid (pure)	Beige granules (pulverized for test)	3·9 (b)
30	Dibenzoyltartaric acid	White powder	2·6 (b)
31	Embonic acid (technical)	Yellow powder	6·2 (b)
32	2,4-Dichloro-5-sulphamoyl-benzoic acid	Beige powder	2·7 (b)
33	2,4-Dichloro-5-chloro-sulphonylbenzoic acid	Beige powder	2·1 (b)

None	26·33	9·33 [4]	—	VI
30 sec	23·50	6·50 [4]	—	VI
4 sec	21·67	6·83 [4]	—	VI
None	28·00	2·17 [4]	—	I
30 sec	22·67	0·83 [4]	—	I
4 sec	22·33	1·17 [4]	—	I
None	52·00	36·50 [7]	—	SvI
30 sec	45·00	34·00 [7]	—	SvI
4 sec	42·67	15·83 [7]	< 15 (4)	SvI
None	16·67	4·17 [4]	—	I
30 sec	16·83	0 [4]	—	I
4 sec	9·33	0·83 [2]	—	SII
None	14·67	4·33 [2]	—	SII
None	108·33§ 92·33	—	—	EI
30 sec	86·67 81·67	—	—	EI
4 sec	71·17	87·50 [7]	—	EI
None	28·67	5·67 [4]	—	VI
30 sec	32·50	1·50 [7]	< 15 (6)	VI
4 sec	14·00	0·33 [2]	—	SII
None	16·67	1·67 [4]	—	I
30 sec	11·67	4·17 [2]	—	SII
None	15·00	0 [4]	—	SII
None	12·00	2·50 [2]	—	SII
None	18·00	2·33 [4]	—	I
30 sec	13·33	1·67 [2]	—	SII
None	25·17	0·33 [4]	—	I
30 sec	22·83	0 [4]	—	I
4 sec	17·83	0 [4]	—	I
None	27·50	9·00 [4]	—	VI
30 sec	22·00	1·17 [4]	—	I
4 sec	18·33	0 [4]	—	I
None	38·50	6·00 [7]	< 15 (6)	VI
30 sec	29·50	4·00 [4]	—	I
4 sec	20·33	2·00 [4]	—	I
None	34·67	11·00 [7]	> 30 (1)	SvI
30 sec	22·50	0 [4]	—	I
4 sec	17·33	0 [4]		I
None	93·00§ 73·00	—	—	EI
30 sec	72·50 70·83	—	—	EI
4 sec	45·33	45·33 [7]	—	SvI

[*contd.*]

Table 3—*continued*

Serial no.	Test material	Appearance	Approx. pH*
34	Ammonium isethionate	Small white crystals	5·2 (b)
35	1,6-Dibromohexane	Colourless liquid	5·2 (a)
36	Caprylyl cystinic acid	White powder	3·2 (b)
37	1-Palmitoyl-4-palmitoyl oxyproline	Beige powder	4·7 (b)
38	Palmitoylcystinic acid	White powder	4·4 (b)
39	*o*-Aminophenol	Beige crystals (pulverized for tests)	7·1 (b)
40	*p*-Aminophenol	White powder	7·3 (b)
41	Sodium fluosilicate	White powder	3·5 (b)
42	Copper nitrate	Blue crystals (pulverized for test)	1·2 (b)
43	Ammonium ferrioxalate	Green crystals (pulverized for test)	5·7 (b)
44	Aluminium nitrate	White crystals (pulverized for test)	0·8 (b)
45	Oxalic acid	White powder	1·0 (b)

Rinsing procedure†	AOI‡	MOI‡	IOI‡	Classification
None	14·00	3·17 [2]	—	SII
None	16·33	0 [4]	—	I
30 sec	14·33	0 [2]	—	SII
None	38·67	26·17 [7]	> 30 (1)	SvI
30 sec	32·50	12·33 [7]	> 30 (1)	SvI
4 sec	33·67	5·33 [7]	⩽ 15 (6)	VI
None	31·67	1·00 [7]	< 15 (6)	VI
30 sec	23·17	3·33 [4]	—	I
4 sec	17·50	0 [4]	—	I
None	12·33	4·17 [2]	—	SII
None	9·83	2·33 [2]	—	SII
None	9·33	1·67 [2]	—	SII
None	62·33§	62·33 [7]	—	EI
	59·00	59·00 [7]		
30 sec	37·00	33·00 [7]	—	SvI
4 sec	35·17	14·83 [7]	> 30 (2)	SvI
None	99·50§	—	—	EI
	86·17			
30 sec	69·83	69·83 [7]	—	EI
4 sec	74·83	74·83 [7]		
	71·50	71·50 [7]	—	EI
None	30·00	22·67 [4]	—	VI
30 sec	15·83	0 [40]	—	I
4 sec	22·00	0 [4]	—	I
None	60·50§	60·50 [7]	—	EI
	57·83	57·83 [7]		
30 sec	32·33	16·83 [7]	> 30 (1)	SvI
4 sec	15·50	1·83 [4]	—	I
None	107·67§	—	—	EI
	91·67			
30 sec	76·17	76·17 [7]	—	EI
	70·17	70·17 [7]		
4 sec	35·67	17·33 [7]	> 30 (1)	SvI

46	Cyclopentanone	Colourless liquid	5·0 (a)	None	54·33	43·67 [7]	> 30 (4)	SvI
				30 sec	49·67	17·50 [7]	> 30 (1)	SvI
				4 sec	54·66	20·67 [7]		SvI
47	o-Diethoxybenzene	Colourless liquid	—	None	16·17	0 [4]	—	I
				30 sec	13·00	1·00 [2]	—	SII
48	Iminophenacyl thiazolidine bromhydrate	Light beige powder	3·5 (b)	None	18·00	0·33 [4]	—	I
				30 sec	15·50	0 [4]	—	I
				4 sec	11·33	0·33 [2]	—	SII
49	p-Phenetidine	Light brown liquid	10·8 (a)	None	41·67	0·33 [7]	≤ 15 (6)	VI
				30 sec	34·33	6·33 [7]	≤ 15 (6)	VI
				4 sec	13·33	0 [2]	—	SII
50	Orthovanilline	Yellow chips (pulverized for test)	4·2 (b)	None	42·33	16·33 [7]	> 15 (3)	SvI
				30 sec	20·67	1·83 [4]	—	I
				4 sec	8·67	2·50 [2]	—	SII
51	Mepyramine	Beige powder	9·2 (b)	None	6·00	0 [2]	—	SII
52	Reticulable silica paste in polysiloxane	Greyish paste	5·6 (b)	None	35·00	13·67 [7]	> 15 (3)	SvI
				30 sec	19·33	5·50 [4]	—	VI
				4 sec	14·00	0 [2]	—	SII
53	Polysiloxane resin	Light yellow viscous liquid	4·5 (a)	None	7·50	1·83 [2]	—	SII
54	Polysiloxane resin (30%, w/w, in toluene)	Colourless viscous liquid	7–7·5 (a)	None	35·67	8·50 [7]	< 15 (5)	VI
				30 sec	28·67	15·33 [4]	—	VI
				4 sec	21·00	0 [4]	—	I
55	Silica paste in polysiloxane	Pasty emulsion	10·1 (a)	None	13·33	8·17 [2]	—	I
				30 sec	13·50	8·33 [2]	—	I
				4 sec	9·50	4·00 [2]	—	SII
56	γ-Glycidoxypropyltrimethoxysilane	Colourless liquid	6·9 (a)	None	11·00	2·33 [2]	—	SII

NI = Non-irritant SII = Slightly irritant I = Irritant VI = Very irritant SvI = Severely irritant EI = Extremely irritant

*pH of (a) named substance, (b) its saturated aqueous solution or (c) the organic phase of a 50:50 (v/v) aqueous solution.

†Where appropriate, rinsing was carried out 30 or 4 sec after instillation of the test substance. For details of procedure see Experimental section.

‡AOI = acute ocular irritation index, which was the 1-hr MOI in the case of the irritant and very irritant substances, except nos 18, 43 and 54 (all 24-hr), and was the 24-hr MOI for the severely and extremely irritant substances, except nos 32 and 50 (the 1-hr and 48-hr MOI, respectively). MOI = mean ocular irritation index after the number of days indicated in square brackets. IOI = individual ocular irritation index on day 7 in the number of rabbits indicated in brackets.

§Where severity of the lesions prevented an accurate reading, two values were calculated, a minimum taking all impossible readings as 0 and a maximum assuming the highest possible score for all such readings. The maximum value was used for the irritation classification.

reached at 1 hr, those for nos 18, 43 and 54 occurred at 24 hr. For these materials the 1-hr MOIs were 24·50, 27·17 and 26·00 compared with the AOIs (Table 3) of 26·33, 30·00 and 35·67, respectively. The AOI for four materials classed as very irritant (nos 18, 24, 30 and 43) did not exceed 30·00 but the MOI at day 4 was too high for a classification of irritant. With most of the 23 irritant/very irritant substances the lesions were reversible by day 7, giving an MOI below 5 at that time, and with all the rest, except ammonium ferrioxalate (no. 43), the lesions were reversible by day 14.

Tests with rinsing. These 23 substances were retested with rinsing 30 sec after instillation, and on the 16 samples that still proved irritant, a third test was conducted involving rinsing after 4 sec. Rinsing at 30 sec reduced the degree of irritation for only about half of the compounds—often those with an irritation index near the upper or lower limit of the range for their classification—and only prevented the development of corneal opacity with two compounds (nos 28 and 47). For eight of the 11 samples that did not change their classification group when rinsed out after 30 sec, rinsing at 4 sec proved useful. Of these eight (nos 13, 16, 21, 24, 48, 49, 54 and 55), three caused no corneal opacity. However, rinsing at 4 sec entailed no further change of classification for substances for which rinsing at 30 sec had been effective.

Severely irritant and extremely irritant test substances

Tests without rinsing. Of the 15 tests substances falling into this category, six (nos 20, 32, 36, 46, 50 and 52) in addition to ethanol (no. 5), the reference material, were classified as severely irritant. These substances had an AOI below 60 but did not meet the 7-day MOI and AOI limitations (and therefore the reversibility requirements) prescribed for the very irritant category. In contrast to the irritant and very irritant substances (see above), the AOI for these substances was the 24-hr MOI except for dichloro-2,4-sulphamoyl-5-benzoic acid (no. 32), which attained the AOI at 1 hr (34·67 *v.* the 24-hr MOI of 28·00) and orthovanillin (no. 50) with MOI values of 40·33, 37·00 and 42·33 (the AOI) at 1, 24 and 48 hr, respectively.

The lesions caused by the severely irritant substances were less readily reversible than those in the very irritant category. The MOI at day 14 or day 21 was below 10 for only three of the seven samples (nos 32, 50 and 52, the latter a silica paste in polysiloxane); isolated areas of corneal opacity persisted at both of these times after treatment with these three substances and to a greater degree after application of the other four.

Of the eight substances classified as extremely irritant (nos 8, 14, 23, 33, 41, 42, 44 and 45), three (nos 14, 41 and 44) had an AOI below 80 but failed to meet the day-7 criteria for severely irritant substances. With most of these substances, the lesions in the eye and related structures were so severe that correct reading was sometimes impossible. In these cases, two calculations were made to give a minimum value, based on the assumption that all the impossible readings were negative (= 0), and a maximum value, derived from assuming the highest possible score for each of these readings. Of course the irritation classification was determined from the second value.

The animals treated with six of the substances in this group were killed after day 7, because of the severity of the lesions. Promethazine (no. 14) and aluminium nitrate (no. 44) produced relatively less severe lesions, however, and in these cases examinations on day 14 and day 21 demonstrated that the effects were slightly reversible with promethazine but not with aluminium nitrate.

Tests with rinsing. Rinsing at 30 sec changed the classification for only seven (nos 5, 14, 32, 41, 44, 50 and 52) of the 15 substances. Only three of these (nos 14, 41 and 44) were in the extremely irritant category when instilled without rinsing. In these cases the irritation remained relatively significant, with corneal opacity persisting for at least the first few days following instillation. However, with some substances (nos 5, 32, 50, 52, 14, 44, 36 and 46) rinsing reduced the severity of the eye lesions and even effected a significant reversibility by day 14 or 21, although for nos 36 and 46 rinsing at 30 sec did not change the classification (nor for the latter did rinsing at 4 sec).

Rinsing 4 sec after instillation promoted a clearer regression of the lesions and resulted in a change or further change in the classification of seven substances (nos 14, 33, 36, 44, 45, 50 and 52). In contrast to some observations on the irritant/very irritant groups, rinsing did not eliminate the risk of corneal opacity. However it did lead to a significant reduction in the severity of the eye lesions, except in three (nos 8, 23 and 42) of the eight substances that were considered to be extremely irritant when instilled without rinsing.

Influence of pH

All four test samples (one liquid and three solids) with a pH below 2 were classified as extremely irritant (Table 3). However the results were more variable for the four solids with a pH between 2 and 3 (nos 19, 30, 32 and 33, which were classified, respectively, as irritant and very, severely and extremely irritant without rinsing). Three of the test substances with a pH in the 10–11 range were very irritant, sometimes even after rinsing, and one was extremely irritant. The fifth, a silica emulsion at pH 10·1 (no. 55) was classed as irritant. Samples with a pH of about 7 were generally among the least irritant, although numbers 31 and 54 (pH 6·2 and 7–7·5, respectively) were both very irritant.

Effect of alternative (OECD) procedure

Recalculation of the AOI values for the 56 test substances on the basis of the readings taken (without rinsing) at the observation times specified in the OECD protocol (only 1, 24 and 72 hr after instillations) gave, in 16 cases, a less severe classification than was obtained using the AFNOR protocol, although the same scale was used for both assessments. Thus, nos 6 and 55 were classed as irritant by the AFNOR method but slightly irritant by the OECD method, 18, 24, 30 and 43 as very irritant by AFNOR and as irritant by OECD, 5, 20, 32, 36, 46, 50 and 52 as severely irritant by AFNOR and as very irritant by OECD and 14, 41 and 44 as extremely irritant by AFNOR and as severely irritant by OECD.

Similarly when the groups were randomly divided and the MOI derived from the scores for all the observation times was calculated first for three rabbits and then for the other three, the classification for 25 of the test materials differed from that deduced from the whole group of six animals. These 26 materials were numbers 5, 6, 10, 15, 16, 19, 21, 24, 26, 31, 32, 35–38, 41, 43–46 and 48–52.

DISCUSSION

Influence of lesion reversibility on assessment of irritancy

The major difference between the AFNOR and the other two protocols is concerned with the expression and interpretation of results, since only the AFNOR protocol gives guidance on the scale for interpreting results (Table 1). Another important difference between the AFNOR, Cosmetics and OECD protocols lies in the observation times specified.

The Kay & Calandra (1962) method, modified by Guillot (1978 & 1979) to facilitate interpretation, takes into account not only the intensity of the reaction at a given time but also the reversibility and the frequency of the visible lesions. Adapting this scale to the OECD protocol by discounting the limitations concerned with the reversibility of the lesions led to nearly a third of the samples being classified in a less severe category than that indicated by the AFNOR procedure.

Influence of rinsing

Although the situation likely to be encountered in accidents involving the human eye may be reflected more realistically by rinsing 60 sec after instillation (AFNOR protocol) than by the shorter intervals (30 and 4 sec) recommended in the OECD protocol (Table 1), we chose to rinse 30 sec after instillation and, where appropriate, also after an interval of only 4 sec. Our own experience, confirmed by the work of Davies, Kynoch & Liggett (1976) on sodium lauryl sulphate, has shown that rinsing 10 sec after instillation is the most effective procedure and that, on the whole, there is little difference between the effects of rinsing 30 sec or several minutes after instillation of the test substance.

The studies reported here confirmed that rinsing was often more effective at 4 sec than at 30 sec after the instillation. However, the irritancy classification assigned to a substance was not necessarily altered when reactions were noticeably less severe or even when a lesion such as corneal opacity was eliminated altogether. Moreover, while rinsing was generally followed by a rapid reduction or even reversal of the injuries, it was found in another study on a product in the slightly irritant category that rinsing appeared to promote a slight increase in the irritation, especially the conjunctival enanthema (Guillot, 1978).

Advantage of using six rabbits

The considerable number of test substances (25 out of 56) that appeared in a different irritancy category when the MOI was calculated on the readings from three rather than six animals or from one set of three animals rather than the other indicated that it is essential to carry out ocular irritation tests in groups of six rabbits, if reliable results and good reproducibility are to be attained.

Influence of pH

The high degrees of irritancy associated with substances with a pH below 2 or above 10 confirmed the recommendation in the AFNOR and OECD protocols that acidic (pH <2) substances should not be tested and, although no material with a pH as high as 11·5 was tested, indicated some support for the similar recommendation on strongly alkaline (pH >11·5) substances (Table 1).

Influence of physical state

No consistent or significant differences were noted between the results obtained with powders, granules or pulverized crystals and those obtained with liquids, semi-liquids or pastes. However, when powders were tested, a slight corneal opacity was sometimes caused by mechanical irritation of the eyeball. We considered that this development depended on the hardness of the test material. This observation has been made with other slightly irritant products, including certain cosmetics (Guillot, Giauffret & Martini, 1979; Guillot, Giauffret, Martini *et al.* 1979, 1980 & 1982; Guillot, Martini & Giauffret, 1977).

Handling precautions

We consider that precise rules for protective measures, such as the wearing of glasses, should be recommended for the handling of any substance that is classified as extremely irritant in the ocular irritation test in the rabbit and also for substances that remain in the severely irritant category in tests involving rinsing 30–60 sec after the instillation. On this basis, such recommendations would apply to 11 of the 56 materials studied, eight (nos 8, 14, 23, 33, 41, 42, 44 and 45) in the former category and three (nos 20, 36 and 46) in the latter.

Conclusions

This comparative study has established (a) that groups of six rabbits are necessary for obtaining reliable results and good reproducibility, (b) that 60 sec appears to be a realistic choice for the time between instillation of the test material and rinsing, bearing in mind the time required to reach a water supply point in the event of accidental contamination of the eye and the need for an adequate margin of safety, and (c) that for an irritant test substance, observation of the reversibility of the lesions over 2–3 wk is required for the assessment of its corrosive potential. Moreover, our experience has shown that the various ocular irritation indices are not a particularly helpful guide in themselves; a graduated scale of irritancy taking into account both these indices and the reversibility of the lesions seems a preferable basis for the interpretation of results.

We consider that the AFNOR protocol best meets these criteria and is sufficiently precise to justify the expectation that different laboratories testing the same compound would draw identical conclusions. Moreover this protocol reflects more closely than the others the common practical conditions of handling. We anticipate that adoption of this protocol would

assist manufacturers in recommending appropriate precautions in the use of their products.

All three protocols depend on subjective readings (Weil & Scala, 1971) of macroscopic changes. The laboratory carrying out the trials must therefore exercise additional controls to ensure the reproducibility and reliability of its assessments. At IFT, technicians who are solely responsible for applying the test substances and reading the irritation (or allergy) studies always conduct the examinations 'blindly', scores obtained at preceding observations being unknown to them. The best qualified staff undertake the most important readings (such as those at 1 and 24 hr). To check that all the technicians are reading the lesions in the same way, a control is arranged monthly; they all examine the same animals and compare their results.

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N-NITROSO-*N*-METHYLDODECYLAMINE AND *N*-NITROSO-*N*-METHYLTETRADECYLAMINE IN HOUSEHOLD DISHWASHING LIQUIDS*

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Abstract—Eleven household dishwashing liquids and four household surface cleaners were analysed for *N*-nitroso-*N*-methyldodecylamine and *N*-nitroso-*N*-methyltetradecylamine by gas chromatography with detection using a Thermal Energy Analyzer. Both nitrosamines were found in three of the dishwashing detergents and one of the surface cleaners. [1-^{14}C]-*N*-Nitroso-*N*-methyldodecylamine was used to determine recoveries, which were between 65 and 88%. Levels of *N*-nitroso-*N*-methyldodecylamine ranged from 112 to 661 ppb and those of *N*-nitroso-*N*-methyltetradecylamine from 46 to 151 ppb. A simple method was developed to screen the products for *N*,*N*-dimethyldodecylamine-*N*-oxide, a surfactant ingredient suspected of being the source of these nitrosamines. By application of this method it was established that all of the products formulated with this amine oxide contained these two nitrosamines, whereas in products that did not contain this ingredient, these nitrosamines were not detected.

INTRODUCTION

The methylalkylnitrosamines *N*-nitroso-*N*-methyldodecylamine (NMDDA) and *N*-nitroso-*N*-methyltetradecylamine (NMTDA) have been detected in hair-care products formulated with the ingredient *N*,*N*-dimethyldodecylamine-*N*-oxide (lauramine oxide) (Hecht, Morrison & Wenninger, 1982). Lauramine oxide and related alkylamine oxides also find widespread use in light-duty liquid dishwashing detergents and surface cleaners (Strauss, Waddams & Kamatari, 1980) and might therefore be a source of nitrosamine contamination of these products. Since NMDDA and NMTDA are both carcinogenic in laboratory animals (Althoff & Lijinsky, 1977; Cardy & Lijinsky, 1980; Ketkar, Althoff & Lijinsky, 1981; Lijinsky, Saavedra & Reuber, 1981; Lijinsky & Taylor, 1975 & 1978) it is important to establish whether household cleaning products constitute a source of human exposure to these nitrosamines. In this study we have analysed several popular dishwashing liquids and liquid cleaners for household use and have demonstrated that NMDDA and NMTDA are present in some of these products.

Many of the products analysed contained no detectable NMDDA or NMTDA. Since in most cases the products did not list their ingredients, we did not know which products were formulated with lauramine oxide and which were not. To test our hypothesis that only those products formulated with lauramine oxide would contain the nitrosamines we developed a simple method to screen for the presence of lauramine oxide.

*This publication is dedicated to the founder of the American Health Foundation, Dr Ernst L. Wynder, on the occasion of the 10th anniversary of the Naylor Dana Institute for Disease Prevention.

Abbreviations: GC = Gas chromatography; MS = mass spectrometry; NMDDA = *N*-nitroso-*N*-methyldodecylamine; NMTDA = *N*-nitroso-*N*-methyltetradecylamine; TEA = Thermal Energy Analyzer.

EXPERIMENTAL

Apparatus. Gas chromatography (GC) with nitrosamine-selective detection was performed at an oven temperature of 210°C using a Hewlett-Packard Model 700 gas chromatograph connected by means of the Brunnemann modification to a Model 543 Thermal Energy Analyzer (TEA, Thermo Electron Corp., Waltham, MA; Brunnemann & Hoffmann, 1981). The GC column was a 12 ft × 2 mm ID glass tube filled with 10% UCW-982 on Gas-Chrom Q 80/100 from Applied Science Laboratories, Inc. (State College, PA). GC for the lauramine oxide test was done with a Hewlett-Packard Model 5710A gas chromatograph equipped with a flame-ionization detector and a 10 ft × 2 mm ID glass chromatography column packed with 3% OV-101 on Gas-Chrom Q 100/120, also from Applied Science Laboratories, Inc. The oven was temperature programmed to hold at the initial temperature of 85°C for 4 min and then increase by 4°C/min until it reached 150°C. The injection port and detector were both at 250°C. Gas chromatography–mass spectrometry (GC–MS) was accomplished with a Hewlett-Packard Model 5982A mass spectrometer using this same GC column. For photolysis of nitrosamines, a Model UVSL-25 Mineralight Lamp from Ultraviolet Products, Inc. (San Gabriel, CA) was operated at 366 nm. A Beckman LS-9000 Liquid Scintillation System (Beckman Instruments, Inc., Irvine, CA) was used for the liquid scintillation counting.

Materials. Cleaning products were purchased in retail stores and are representative of popular brands now available. *N*-Methyldodecylamine was obtained from Pfaltz and Bauer. Inc. (Stamford. CT) and *N,N*-dimethyldodecylamine from Alfa Products (Danvers. MA) and were used without purification. AG1-X8 20-50 Mesh analytical grade anion exchange resin. from Bio-Rad Laboratories (Richmond. CA). was used in the hydroxide form. The syntheses of *N*-nitroso-*N*-methyldodecylamine. *N*-nitroso-*N*-methyltetradecylamine and [1-^{14}C]-*N*-nitroso-*N*-methyldodecylamine) [1-^{14}C]NMDDA) have been described (Hecht *et al.* 1982). 1-Dodecene was kindly provided by Dr J. Morrison of the American Cyanamid Company. Silica-gel was Baker Analyzed grade. 40–140 mesh. from Baker Chemical Co. (Phillipsburg. NJ). and thin-layer chromatography (TLC) plates were 0·25 mm silica-gel 60 F-254 from EM Laboratories (Elmsford, NY). Scinti-Prep 2 for LSC and ascorbic acid were from Fisher Scientific Company (Fair Lawn. NJ). All solvents were of spectral purity.

Quantitative analysis for NMDDA and NMTDA in household cleaning products. The analytical procedure, which involves the use of ascorbic acid to inhibit artefactual formation and [1-^{14}C]NMDDA as internal standard. was identical to that described for analysis of hair-care products (Hecht *et al.* 1982). NMDDA and NMTDA were determined using the GC–TEA. correcting for recoveries of [1-^{14}C]NMDDA.

Photolysis experiments. The procedure for performing photolysis of these samples is also described in Hecht *et al.* 1982.

Analysis by GC–MS. Two 10-g batches of one product (Product A) were prepared as for the quantitative analysis. The resulting solutions were combined and applied to four 20 × 20 cm TLC plates and developed in chloroform *v*. NMDDA standard. The bands corresponding to NMDDA were scraped. combined and extracted by sonication in methanol. This extract was filtered and evaporated to dryness with a rotary evaporator at 45°C. The residue was redissolved in chloroform and analysed by GC–MS. The GC oven temperature was programmed from 120 to 250°C at 1°/min.

Lauramine oxide test. A 5-g sample of product was stirred for 3 min with 100 ml 70% aqueous ethanol adjusted to pH 9–10 with 10 N-NaOH. A 25-g portion of AG1-X8 anion exchange resin in hydroxide form. damp with methanol. was weighed out and added to the mixture. After stirring for 30 min. the mixture was filtered and concentrated to dryness by rotary evaporation at 55°C. The residue was dissolved in 0·5–2·0 ml of MeOH. Aliquots of these solutions were then analysed by GC.

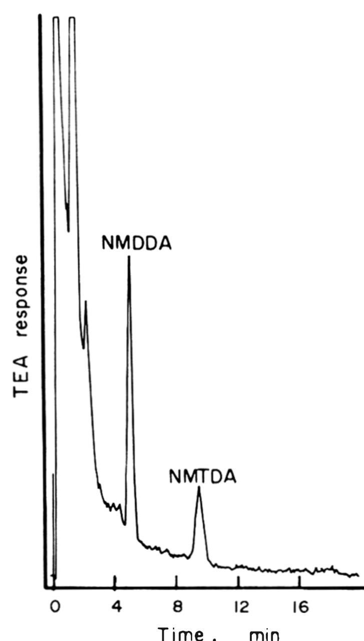

Fig. 1. GC–TEA chromatogram of NMDDA and NMTDA in a household dishwashing liquid.

RESULTS

Figure 1 shows a GC–TEA chromatogram typical of a product containing NMDDA and NMTDA. In each case where positive values are reported, the indicated components coeluted with standard NMDDA and NMTDA when coinjected. Furthermore. each of the 'positive' samples exhibited a loss of TEA response for NMDDA and for NMTDA when reinjected following irradiation with ultraviolet light, a characteristic reaction of *N*-nitroso compounds (Krull, Goff, Hoffman & Fine. 1979). One sample (product A, sample 2) was prepared on a larger scale for analysis by GC–MS. and the identities of NMDDA and NMTDA were confirmed by comparison to standard spectra (Hecht *et al.* 1982).

To test the reproducibility of this analytical procedure, a replicate analysis of one sample (product A, sample 1) was performed; the results are shown in Table 1. To test for possible artefact formation in the analytical procedure despite the use of ascorbic acid to inhibit nitrosation, one product (product A, sample 1) was analysed after the addition of 1 mg each of *N*-methyldodecylamine and sodium nitrite. The amounts of NMDDA and NMTDA found were within the limits of experimental error shown in Table 1. Thus, the levels of NMDDA and NMTDA reported here cannot be explained by artefactual formation of the compounds.

Products were also analysed to establish whether they were formulated with lauramine oxide. The lauramine oxide test was considered positive when the chromatogram indicated the presence of both 1-dodecene, a pyrolysis product of lauramine oxide. and

Table 1. *Reproducibility of analyses for NMDDA and NMTDA in household dishwashing liquids*

Analysis no.	Recovery of [^{14}C]NMDDA (%)	NMDDA (ppb)	NMTDA (ppb)
1	86	269	93
2	96	292	101
3	82	307	117
4	86	274	66
5	83	302	66
Mean	87	289	89
Standard deviation	6	17	22

Table 2. *NMDDA and NMTDA in household cleaning products*

Product code	Type	Concentration (ppb)*		Lauramine oxide test
		NMDDA	NMTDA	
A1†	LDWD	289	89	+
A2†	LDWD	661	151	+
B	LDWD	254	67	+
C	LDWD	471	108	+
D	LDWD	ND	ND	–
E	LDWD	ND	ND	–
F	LDWD	ND	ND	–
G	LDWD	ND	ND	–
H	LDWD	ND	ND	–
J	LDWD	ND	ND	–
K	LDWD	ND	ND	–
L	LDWD	ND	ND	–
M	SC	112	46	+
N	SC	ND	ND	–
P	SC	ND	ND	–
Q	SC	ND	ND	–

LDWD = Liquid dishwashing detergent SC = Surface cleaner with mild abrasive
ND = Not detected (detection limits; NMDDA, 15 ppb; NMTDA, 20 ppb
*[1-^{14}C]NMDDA was used as internal standard.
†Two different bottles of product A.

N,N-dimethyldodecylamine (DMDDA), the precursor in the industrial synthesis of lauramine oxide, at levels greater than 100 ppm (Lew, 1964; Strauss *et al.* 1980). Under these GC conditions, the retention times of 1-dodecene and DMDDA were 13·9 and 45·0 min. One sample was analysed under these GC conditions by GC–MS. The mass spectra of the compounds eluting at the retention times of 1-dodecene and DMDDA were identical to those of the standards.

Table 2 lists the results of the analyses of eleven liquid dishwashing detergents and four household surface cleaners. Values for concentrations of NMDDA and NMTDA are corrected for recoveries of [1-^{14}C]NMDDA, which ranged from 65 to 88%. Table 2 also indicates which products were found to be formulated with lauramine oxide. All three dishwashing liquids and the surface cleaner that were found to be formulated with lauramine oxide were also found to contain NMDDA and NMTDA. Levels of NMDDA ranged from 112 to 661 ppb and those of NMTDA from 46 to 151 ppb. None of the products without lauramine oxide contained detectable levels of NMDDA or NMTDA.

DISCUSSION

The results of this study demonstrate that certain household cleaning products are contaminated with the methylalkylnitrosamines NMDDA and NMTDA. Furthermore, this study shows that in every case where lauramine oxide was an ingredient, the product contained NMDDA and NMTDA and in every case where lauramine oxide was not an ingredient the nitrosamines were not detectable. One household surface cleaner and three dishwashing liquids, the only products formulated with lauramine oxide, were found to contain NMDDA and NMTDA. Mass spectral confirmation of the identities of the nitrosamines was obtained from one sample, but all four products gave GC–TEA traces similar to Fig. 1, with positive responses for both NMDDA and NMTDA. These peaks increased on coinjection with the standard compounds and decreased after photolysis. Eleven other products, not formulated with lauramine oxide, gave no TEA responses for either NMDDA or NMTDA.

Lauramine oxide is one of a class of compounds known as fatty amine oxides, that are commercially useful due to their surfactant properties. NMDDA and NMTDA have previously been detected as impurities in some hair-care products formulated with lauramine oxide (Hecht *et al.* 1982). However, by far the greatest use of fatty amine oxides is in light-duty liquid dishwashing detergents. One source estimates that 30 million pounds of alkyldimethylamine oxide was consumed in the United States in 1977, 77% of which was incorporated into light-duty liquid dishwashing detergents (Bradley, 1978). Although commercial preparations of amine oxides are generally mixtures of different alkyl chain-lengths, lauramine oxide typically predominates, usually accounting for at least half of the amine oxide on a mass basis (Turan & Gibson, 1981). In the light of this widespread use of lauramine oxide, this study indicates that household dishwashing liquids, in addition to hair-care products, must be considered as a source of human exposure to NMDDA and NMTDA.

The source of this nitrosamine contamination is not yet certain. Amine oxides have previously been shown to yield nitrosamines when treated with a suitable nitrosating agent. Smith & Loeppky (1967) found that *N*-nitrosodibenzylamine was formed from tribenzylamine-*N*-oxide and nitrous acid. The reaction of trimethylamine-*N*-oxide and sodium nitrite in aqueous acid gives *N*-nitrosodimethylamine (Lijinsky, Keefer, Conrad & Van de Bogart, 1972; Lijinsky & Singer, 1973). Also, in model studies, we found NMDDA

when sodium nitrite was reacted with a commercial preparation of lauramine oxide (Hecht & Morrison, unpublished data, 1980). Thus, it is possible that lauramine oxide in these detergent products reacts with some nitrosating agent to yield NMDDA. However, it is also possible that other species are the precursors of the nitrosamines.

Amine oxides are produced industrially by the oxidation of dimethylalkylamines with hydrogen peroxide. Thus, these tertiary amines, or secondary amine impurities, might undergo nitrosation. This is particularly interesting in the light of a recent study showing the formation of *N*-nitrosodiethanolamine from hydrogen peroxide and diethanolamine (Ong, Rutherford & Wich, 1981).

When administered by gavage to Sprague–Dawley or Fischer rats, NMDDA gave 100% incidence of bladder tumours (Lijinsky & Taylor, 1975; Lijinsky & Taylor, 1978). This compound also induced primarily bladder tumours in Syrian hamsters and liver tumours in guinea-pigs (Althoff & Lijinsky, 1977; Cardy & Lijinsky, 1980). Subcutaneous administration of NMDDA in European hamsters induced neoplasms of the lung and bladder (Ketkar *et al.* 1981). NMTDA also induces bladder tumours in Fischer rats, but apparently is not as potent a carcinogen as NMDDA in this species (Lijinsky *et al.* 1981). The skin penetrability of these compounds has not been studied.

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QUANTITATIVE ANALYSIS OF CATECHOL AND 4-METHYLCATECHOL IN HUMAN URINE*

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Abstract—A method was developed for the quantitative analysis of catechol and 4-methylcatechol in human urine. [U-^{14}C]Catechol was used as an internal standard. Urine was treated with β-glucuronidase and sulphatase, acidified and extracted with ether. The ether extract was silylated and analysed by glass capillary gas chromatography. Catechol and 4-methylcatechol occurred in urine primarily as conjugates. Levels of catechol and 4-methylcatechol in the urine of nonsmokers on unrestricted diets were $10 \pm 7{\cdot}3$ (mean $\pm$ 1 SD) and $3{\cdot}4 \pm 2{\cdot}3$ mg/24 hr, respectively. Nonsmokers on uniform restricted diets, in which the intake of plant-derived products was limited, excreted $4{\cdot}4 \pm 1{\cdot}2$ mg catechol and $8{\cdot}1 \pm 1{\cdot}7$ mg 4-methylcatechol/24 hr. Smokers on the same restricted diet excreted $6{\cdot}8 \pm 3{\cdot}0$ mg catechol and $6{\cdot}1 \pm 2{\cdot}6$ mg 4-methylcatechol/24 hr. These results indicate that diet is a major factor in determining urinary catechol levels and that the contribution of smoking is comparatively small. Catechol and 4-methylcatechol appear to have different dietary precursors.

INTRODUCTION

Catechol (1,2-benzenediol) is an important constituent of cigarette smoke. It occurs at relatively high levels in the particulate matter of smoke and is co-carcinogenic with benzo[*a*]pyrene on mouse skin (Brunnemann, Lee & Hoffmann, 1976; Hecht, Carmella, Mori & Hoffmann, 1981; Van Duuren & Goldschmidt, 1976). Catechol may therefore be involved in the tobacco-related cancers, which include cancer of the lung, oral cavity, oesophagus, pancreas and bladder (US Department of Health and Human Services, 1979). Compounds related to catechol also occur widely as dietary constituents. The widespread occurrence of catechol as well as its co-carcinogenic activity prompted us to develop a method for the analysis of catechol in human urine. The presence of catechol as a constituent of human urine has been reported previously, but we are not aware of any quantitative data (Dirmikis & Darbre, 1974; Lewis, Kenyon, Meili & Burlingame, 1979; von Euler & Lishajko, 1959). In the present report, we describe a simple gas-chromatographic method for the quantitative analysis of catechol in human urine. The method was used to analyse the urine of smokers and of nonsmokers on restricted and unrestricted diets. The results indicate that diet is the major factor in determining urinary levels of catechol.

EXPERIMENTAL

Apparatus. Gas–liquid chromatography (GLC) was carried out with a Hewlett-Packard model 5830A gas chromatograph equipped with a model 18835B capillary inlet system, a 30-m SP 2100 glass capillary column (Supelco Inc., Bellefonte, PA), and a flame ionization detector. The gas chromatograph was operated in the split mode with a split ratio of 40:1. The oven was heated with a temperature programme of 80–230°C at 2°/min. The flow rate of He was 1 ml/min. Gas–liquid chromatography–mass spectrometry (GLC–MS) was performed with a Hewlett-Packard Model 5982A instrument using the same column and conditions. Incubations were carried out using a Lab-Line orbital shaker (150 rpm; Lab-Line Instruments Inc., Melrose Park, IL) which was enclosed in a Lab-Line Imperial II incubator. Scintillation counting was carried out using a Nuclear Chicago Corp. (Des Plaines, IL) Isocap 300 system.

Reagents. Ether preserved with butylated hydroxytoluene was obtained from Scientific Products (McGaw Park, IL). Catechol and 4-methylcatechol were obtained from Aldrich Chemical Co. (Milwaukee, WI). Regisil RC-2 [bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane] was obtained from Regis Chemical Company (Morton Grove, IL). β-Glucuronidase type H-1 was purchased from Sigma Chemical Co. (St. Louis, MO). [U-^{14}C]-Catechol (2·5 mCi/mmol) was obtained from New England Nuclear (Boston, MA).

Separation of phenol compounds. Phenol, catechol, resorcinol and hydroquinone were obtained from Fisher Scientific Company, Springfield, NJ and *o*-, *m*- and *p*-cresol, 2,3- and 2,6-dimethylphenol, *m*- and *p*-ethylphenol, *p*-hydroxyanisole and 3- and 4-methylcatechol were obtained from Aldrich Chemical Co., Inc, Milwaukee, WI. Pyrogallol was purchased from ICN/K and K, Life Sciences Group, Plainview, NY. Aliquots (100–300 ng) of these compounds, as their trimethylsilyl derivatives, were separated by glass capillary gas chromatography under the conditions detailed above.

Urine samples. Twenty-four-hour urine samples

*This publication is dedicated to the founder of the American Health Foundation, Dr Ernst L. Wynder, on the occasion of the 10th anniversary of the Naylor Dana Institute for Disease Prevention.

Abbreviations: GLC = Gas–liquid chromatography; GLC–MS = gas–liquid chromatography–mass spectrometry.

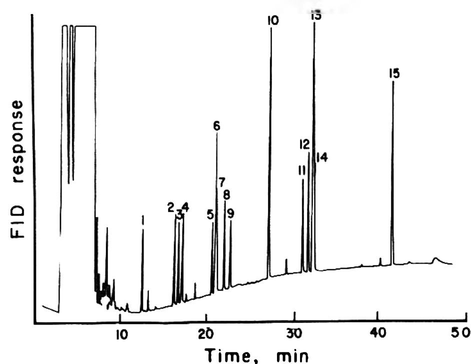

Fig. 1. Separation of some phenolic compounds, as their trimethylsilyl derivatives, by glass capillary gas chromatography (for conditions see Experimental section). The peaks identified were as follows: (1) phenol, (2) *o*-cresol, (3) *m*-cresol, (4) *p*-cresol, (5) 2,6-dimethylphenol, (6) *p*-hydroxyanisole, (7) *m*-ethylphenol, (8) *p*-ethylphenol, (9) 2,3-dimethylphenol, (10) catechol, (11) resorcinol, (12) 4-methylcatechol, (13) 3-methylcatechol, (14) hydroquinone, (15) pyrogallol.

were obtained from 17 healthy male volunteers, who ranged in age from 20 to 56 yr and in weight from 55 to 77 kg.

Urine samples were collected from 11 of the volunteers (all nonsmokers) who ate an unrestricted diet. Seven volunteers who were nonsmokers were given restricted diets for 2 days prior to and during a 24-hr urine collection. Breakfast and lunch were served in the cafeteria of the Naylor Dana Institute for Disease Prevention. Breakfast consisted of a ham and egg sandwich and milk or water. Lunch was a tuna-fish sandwich on the first day, a turkey sandwich on day 2, and a roast beef sandwich on day 3 with milk, water or a non-fruit soft drink. The dinners consisted of broiled chicken with rice, a roll and coffee cake (day 1), spaghetti with tomato sauce and cheese, and chocolate ice cream (day 2), and steak with rice, bread, and cheesecake (day 3). Beverages taken with dinner were restricted to beer, milk, water or a non-fruit soft drink. There was no consumption of fresh fruits, vegetables, coffee, tea, wine or fruit juices. Five of the volunteers who were smokers followed the same restricted diet and smoked a recorded number

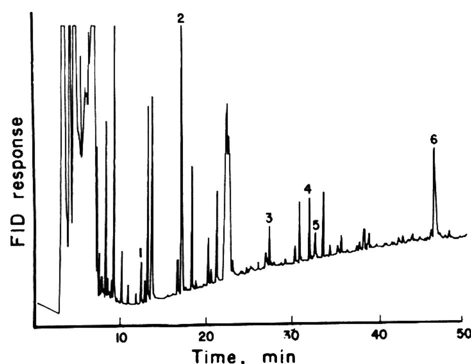

Fig. 2. Gas chromatogram of the catechol fraction of human urine. The peaks were identified by combined GLC–MS as (1) phenol, (2) *p*-cresol, (3) catechol, (4) 4-methylcatechol, (5) hydroquinone, (6) *p*-hydroxyphenylacetic acid.

Table 1. *Replicate analysis of catechols in human urine*

Replicate	Urinary catechols (mg/24 hr)	
	Catechol	4-Methylcatechol
1	16·3	3·4
2	15·8	4·5
3	14·8	3·7
Mean ± 1 SD...	15·6 ± 0·8	3·9 ± 0·6

Urine was obtained from a smoker on an unrestricted diet. The urinary catechols were present predominantly as conjugates, and levels were determined by the isotope dilution method.

of typical US 85-mm non-filter cigarettes for the 2 days prior to and during the day of the 24-hr urine collection.

Analysis of human urine for conjugated and free catechols. Urine was collected in 1-litre polyethylene bottles containing 0·5 g ascorbic acid and 10 ml 1 N-HCl. Samples were processed immediately after the collection. For the analysis for conjugated catechols a 50-ml aliquot was removed, placed in a 125-ml Erlenmeyer flask, and adjusted to pH 5 with 2% aqueous $NaHCO_3$, or 3 N-HCl. [U-^{14}C]Catechol (1 × 10^5 dpm) in 1 ml ether (as an internal standard) and 160,000 U β-glucuronidase (type H-1 containing 7400 U sulphatase activity) were added. The mixture was incubated and continuously shaken overnight at 37°C, and then adjusted to pH 2 with 3 N-HCl and extracted three times with equal volumes of ether. The ether layers were combined, swirled with 15 g Na_2SO_4, decanted, and then dried further with Na_2SO_4. The mixture was filtered through a Buchner funnel and brought to near dryness under reduced pressure at 25°C. The residue was redissolved in CH_3CN and the volume adjusted to 2·0 ml. A 0·1-ml aliquot was used to determine recovery of ^{14}C-activity. A second 0·1-ml aliquot was silylated by heating with 0·2 ml Regisil RC-2 and 0·1 ml CH_3CN in a 1-ml Reactivial (Pierce Chemical Co., Rockford, IL) at 80°C for 1 hr. A 3-μl aliquot of this silylation mixture was analysed by GLC. Peaks were quantified by comparison with standard curves for silylated catechol and 4-methylcatechol, and levels of catechol and 4-methylcatechol in urine were calculated by the isotope dilution method.

For the analysis of urine for free catechols the procedure was identical to that described above except that a 100-ml aliquot of urine was used and the enzymatic hydrolysis was omitted.

RESULTS

An excellent separation of several phenolic compounds, as their trimethylsilyl ethers, was obtained on a glass capillary column as shown in Fig. 1. Of particular interest in relation to this study were the separations of three benzenediol isomers—catechol, resorcinol and hydroquinone—and of 3- and 4-methylcatechol. A typical gas chromatogram of the catechol fraction of human urine is illustrated in Fig. 2. The numbered peaks were identified by their retention times and by GLC–MS as phenol (peak 1), *p*-cresol (2), catechol (3), 4-methylcatechol (4), hydroquinone (5) and *p*-hydroxyphenylacetic acid (6).

Data from a triplicate analysis for catechol and 4-methylcatechol in a 24-hr sample collected from a smoker who was consuming an unrestricted diet are presented in Table 1. Recovery of [U-^{14}C]catechol was in the range 60–80%. The limit of detection was approximately 0·5 ppm, or 0·5 mg of either catechol or 4-methylcatechol per 24-hr urine collection. Analysis of urine that had not been treated with β-glucuronidase and sulphatase revealed that the levels of free catechol represented 1–5% of the total.

Levels of catechol and 4-methylcatechol in the urine of the nonsmoking volunteers on restricted and unrestricted diets are presented in Table 2. Amongst those given the unrestricted diet a wide variation was observed. Catechol levels ranged from 1·2 to 30 mg/24 hr (1·6–32 ppm) and 4-methylcatechol levels ranged from undetectable to 7·2 mg/24 hr (up to 8·7 ppm). In contrast, narrower ranges of values were observed for both catechol and 4-methylcatechol when the diets were restricted and uniform (2·5–5·9 mg catechol/24 hr, 6·6–11 mg 4-methylcatechol/24 hr). Among these nonsmokers the ratio of catechol to 4-methylcatechol was >1 in every sample from donors on the unrestricted diet but was <1 in every sample from donors on restricted diets.

Table 3 presents levels of catechols in the urine of smokers on the same restricted diet as the donors in Table 2. The mean level of catechol (6·8 mg/24 hr) was somewhat higher than that for the nonsmokers on the restricted diet (4·4 mg/24 hr) and the catechol levels for donors 16 and 17 who were heavy smokers were higher than those of any of the other donors on the restricted diet. The mean level of 4-methylcatechol in the urine of the smokers (6·1 mg/24 hr) was slightly lower than that of the nonsmokers on the restricted diet (8·1 mg/24 hr).

Table 2. *Catechols in the urine of nonsmokers consuming unrestricted and restricted diets*

Volunteer no.	Urine volume (litres/24 hr)	Urinary catechols (mg/24 hr): Catechol	Urinary catechols (mg/24 hr): 4-Methyl-catechol
Unrestricted diet			
1	1·80	12	2·7
2	1·25	12	1·5
3	0·74	1·2	ND
4	2·23	4·5	NC
5	0·88	10	3·6
6	2·62	6·3	NC
7	1·48	2·6	ND
8	0·71	9·7	4·1
9	1·02	13	5·8
10	0·93	30	7·2
11	0·52	9·6	4·5
Mean ± 1 SD..	1·29 ± 0·64	10 ± 7·3	3·4 ± 2·3
Restricted diet			
1	0·96	5·9	9·8
2	1·46	4·4	11
3	0·74	2·5	8·0
8	0·67	5·0	6·6
9	1·09	3·6	7·3
10	1·01	5·8	7·7
12	0·53	3·6	6·6
Mean ± 1 SD..	0·92 ± 0·31	4·4 ± 1·2	8·1 ± 1·7

ND = Not detected

NC = Not determined due to interfering peak

Catechols were present predominantly as conjugates and levels were determined by the isotope dilution method.

DISCUSSION

The present method was designed to avoid alkaline conditions, under which catechol is unstable. Urine was collected in acid, and ascorbic acid was added as an antioxidant. The combination of capillary GLC and silylation produced excellent resolution of the phenolic compounds found in urine.

Our choice of the type and amount of enzyme to use in the enzymatic hydrolysis step was based on a previous study in which β-glucuronidase type H-1, which has sulphatase activity, was shown to be efficient in deconjugating catechol in urine samples from people who had voluntarily ingested several milligrams of catechol (Bakke & Scheline, 1969). When we increased the levels of β-glucuronidase type H-1 used in the hydrolysis step, we did not observe any change in catechol levels. Since β-glucuronidase type B-1, without sulphatase, was not efficient in deconjugating catechol, the major conjugate in human urine would appear to be the sulphate (Bakke & Scheline, 1969). However, in the rabbit the major conjugate of catechol is apparently the monoglucuronide (Garton & Williams, 1948).

The results of this study on a limited number of donors indicate that diet is the major factor contributing to catechol levels in human urine. Catechol

Table 3. *Catechols in the urine of smokers on restricted diets*

Volunteer no.	No. of cigarettes* smoked/24 hr	Catechols inhaled (mg/24 hr)†: Catechol	Catechols inhaled (mg/24 hr)†: 4-Methylcatechol	Urine volume (litres/24 hr)	Urinary catechols (mg/24 hr)‡: Catechol	Urinary catechols (mg/24 hr)‡: 4-Methylcatechol
13	6	1·6	0·1	1·69	4·4	4·7
14	7	1·9	0·1	1·15	4·1	2·1
15	15	4·0	0·3	1·25	5·1	8·0
16	27	73	0·6	1·15	11	8·2
17	30	8·1	0·6	1·44	9·4	7·5
Mean ± 1 SD...		4·6 ± 3·0	0·3 ± 0·3	1·34 ± 0·23	6·8 ± 3·0	6·1 ± 2·6

*Typical 85-mm non-filter US cigarette, containing 272 μg catechol and 21 μg 4-methylcatechol/cigarette in the mainstream smoke (Brunnemann, Lee & Hoffmann, 1976).

†Based on standard machine smoking conditions and assuming uniform inhalation among donors.

‡Urinary catechols were present predominantly as conjugates and levels were determined by the isotope dilution method.

levels in the urine of donors who consumed a uniform restricted diet were lower than those of donors whose diet was not restricted and showed markedly less variation between subjects. The restricted diet was composed primarily of meat, poultry and carbohydrates. Coffee and tea were not allowed and consumption of fruit and vegetable products was extremely limited. Thus, these products would appear to contain precursors to urinary catechol. This observation is in agreement with a previous study that demonstrated that excretion of catechols in rat urine was reduced or eliminated when the rats consumed diets free of plant-derived material (Bakke, 1969). Several compounds that occur in plants, including quinic acid, shikimic acid, vanillin and various phenolic acids have been shown to be metabolic precursors of catechol in experimental animals (Booth, Robbins, Masri & DeEds, 1960; Indahl & Scheline, 1973; Scheline, 1968; Strand & Scheline, 1975).

The influence of diet on urinary levels of 4-methylcatechol appeared to be opposite to that observed for catechol. Dietary restriction resulted in an increase in urinary 4-methylcatechol. Apparently, catechol and 4-methylcatechol have different dietary precursors. Because of the relatively low levels of 4-methylcatechol in cigarette smoke, the contribution of smoking to urinary 4-methylcatechol is negligible.

The levels of urinary catechol among smokers on the restricted diet appeared to increase in proportion to the amount of catechol inhaled. However, the amounts of urinary catechol that can be attributed to smoking are insignificant when compared to those found among nonsmokers on normal, unrestricted diets. Thus, catechol derived from tobacco smoke is not likely to be a causative factor in bladder cancer induced by smoking.

Catechol is a potent co-carcinogen with benzo[*a*]-pyrene on mouse skin (Hecht *et al.* 1981; Van Duuren & Goldschmidt, 1976). The co-carcinogenic potential of catechol sulphate or glucuronide to the urinary bladder has not been investigated. Assays to determine whether catechol and its conjugates are co-carcinogenic with urinary bladder carcinogens such as *N*-nitroso-*n*-butyl-*N*-(4-hydroxybutyl)amine and 2-naphthylamine would be appropriate. These studies are necessary in order to assess the potential roles of urinary catechol and 4-methylcatechol as causative factors in human bladder cancer.

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SHORT PAPERS

ENTERAL ABSORPTION AND BIOTRANSFORMATION OF THE FOOD ADDITIVE OCTYL GALLATE IN THE RAT

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Abstract—Following oral administration of ^{14}C-labelled octyl gallate in a single dose of 15 mg/kg to female rats, only 20–30% of the radioactivity administered was detected in the tissues, while 60–80% of the dose was found in the contents of the gastro-intestinal tract up to 12 hr after administration. Isotope dilution analysis demonstrated the presence of the unchanged ester in the tissues. In the liver, the highest concentration of the ester demonstrated was 1·6 μg/g, in a rat killed 10 min after treatment. In the 24 hr following ip administration of labelled octyl gallate, about 91% of the administered radioactivity was recovered. Most of this was in the form of metabolites, only 9% being accounted for as unchanged ester.

Introduction

Octyl gallate (the *n*-octyl ester of 3,4,5-trihydroxybenzoic acid) is a synthetic antioxidant which is added to human food to inhibit the autoxidation of lipids. Although it has been used in this way for a number of years (Council of the European Communities, 1970; Joint FAO/WHO Expert Committee on Food Additives, 1962) there is little information on its fate in mammals. Only van Esch (1955) has described an experiment in which octyl gallate was given to rats. Gallic acid and some unidentified phenolic substances were detected in the animals' excreta but quantitative data on the extent of the absorption and biotransformation were not reported.

To contribute to an evaluation of the safety of octyl gallate as a food additive, we considered it important to study its enteral absorption, distribution and biotransformation.

Experimental

Preparation of test material. [1-^{14}C]Octyl gallate (mol wt 282) was prepared by re-esterification of 1 mmol *n*-[1-^{14}C]octanol with a specific activity of 4·32 mCi/mmol with 2 mmol methyl gallate in the presence of 0·5 mmol hydrochloric acid (= 50 μl conc. hydrochloric acid) at 166°C. The labelled *n*-octanol was kindly provided by Dr P. E. Schulze (Schering AG, Berlin). The melting point of the synthesized ester after recrystallization from diethyl ether was 93–95°C (cf. 94–95°C, Morris & Riemenschneider, 1946); GLC and TLC analyses demonstrated >99·9% chemical and radiochemical purity, respectively. Prior to administration to the rats, the labelled compound was diluted with appropriate amounts of non-labelled octyl gallate to yield a specific activity of 4·1 mCi/mmol (14·5 μCi/mg) for oral administration and of 3·4 mCi/mmol (12 μCi/mg) for ip administration. For administration, the ester was dissolved in Tween 80 (Serva-Technik GmbH, Heidelberg) in a ratio of 1:5 (w/w) and this solution was mixed with physiological saline to yield a final concentration of 1 mg [^{14}C]octyl gallate/100 μl solution. The labelled dose administered orally by means of a stomach tube or injected amounted to 15 mg/kg.

Animals and treatment. Female Wistar rats (from the Zentralinstitut für Versuchstierzucht, Hannover, and weighing 130–160 g) were housed in cages fitted for the collection of faeces, urine and expired air. Urine was collected in a reservoir maintained at −6°C. The rats had unrestricted access to Altromin R-10 diet (Altromin GmbH, Lippe) and tap-water. Doses of 15 mg [^{14}C]octyl gallate were administered orally, by stomach tube, or by ip injection. The rats were killed by decapitation at various times after treatment.

Sampling procedure and isotope measurements. Radioactivity in the tissues was determined after dissolving 40–70-mg tissue samples in Soluene 350 (Packard Instrument Co. Ltd, Downers Grove, IL, USA). Faeces were homogenized in 2–3 vols 0·5 N-perchloric acid and the homogenates were extracted once with an equal volume of diethyl ether and centrifuged. The radioactivity was determined in the organic and in the aqueous layer, and also in the sediment after it had been dissolved in Soluene. Gut contents were obtained by washing out the stomach, small intestine, caecum and colon with diethyl ether. They were processed and assayed for carbon-14 in the same manner as were the faeces. Coloured samples were bleached with hydrogen peroxide prior to the counting of carbon-14.

The content of the unchanged drug in the tissues, gut contents, faeces and urine was determined by isotope dilution analysis. To each of the samples, an appropriate amount of unlabelled octyl gallate was added. Following homogenization in 0·5 N-perchloric

Abbreviations: GLC = Gas–liquid chromatography; TLC = thin-layer chromatography.

Table 1. *Recovery of radioactivity in the rat carcass, gut, expired air and excreta after administration of [^{14}C]octyl gallate in a single oral dose of 15 mg/kg (0·22 mCi/kg)*

Sample	Recovery of radioactivity (% of dose) at			
	10 min*	30 min*	6 hr*	12 hr*
Carcass†	12·7	9·7	7·0	6·4
Gut wall	13·7	12·4	4·0	2·0
Gut contents	74·0	81·0	65·2	61·0
Expired air‡	0·4	1·8	17·3	19·6
Urine‡	0·1	1·1	5·3	6·0
Faeces‡	RND	RND	RND	RND
Total...	100·9	106·0	98·0	95·1

RND = Radioactivity not detected
*Time after treatment; one rat was killed at each time.
†Whole body minus gastro-intestinal tract and its contents.
‡Collected from treatment until death.

acid the samples were extracted exhaustively with diethyl ether and the extracts were evaporated. The extracted [^{14}C]OG was purified by recrystallization in a diethyl ether–*n*-pentane (1:1, v/v) mixture until more than three consecutive samples possessed the same specific activity.

The radioactivity eliminated via the lungs was collected by drawing the expired air through 2 N-methanolic ethanolamine (Kalberer & Rutschmann, 1961). To confirm that the expired radioactivity was [^{14}C]CO_2, the collected label was liberated from the methanolic ethanolamine solution by hydrochloric acid at room temperature and passed in a constant nitrogen stream through three flasks containing *n*-hexane, ethanolamine and aqueous barium hydroxide, respectively.

For the determination of retained carbon-14 the carcass was dissolved in 2 N-methanolic potassium hydroxide (Petroff, Patt & Nair, 1965).

Liquid scintillation counting was performed with a Packard Tri-Carb Model 3380. The radioactivity was determined after addition of a Triton X-100–scintillator toluene (1:2, v/v) mixture (Patterson & Greene, 1965) to the Soluene solutions, to aliquots of the organic and aqueous layers from homogenate extractions or to urine. Counting efficiency was determined by adding [^{14}C]hexadecane (Amersham Buchler GmbH & Co. KG, Braunschweig) as an internal standard.

Results

In order to find out whether octyl gallate was absorbed enterally, rats were given a single oral dose of the ^{14}C-labelled ester. Analyses showed the tissues, gastro-intestinal tract, faeces, urine and expired air of the animals to contain carbon-14 (Table 1). Expired radioactivity was not trapped in *n*-hexane but was trapped quantitatively in both ethanolamine and barium hydroxide, confirming that all the label was present as $^{14}CO_2$. Since this indicated metabolic alteration of the ester, isotope dilution analyses were performed to distinguish between the parent compound and its breakdown products. Repeated recrystallization of unlabelled octyl gallate added to the liver and extracted after homogenization of the tissue led to nearly constant specific activity values (Table 2), indicating the presence of unchanged labelled ester in this organ. From the figures in Table 2 it was calculated that the liver content of [^{14}C]octyl gallate determined 10 min after administration amounted to 1·6 μg/g wet tissue. Unchanged [^{14}C]octyl gallate was also detected in adipose tissue, blood and the wall of the gastro-intestinal tract (Table 3). The label not attributable to the unchanged compound was interpreted as representing metabolites of the ester.

Since octyl gallate had been shown to be absorbed from the gut, it was of interest to estimate the extent of its biotransformation. Rats were given 15 mg [^{14}C]octyl gallate/kg ip and were killed after 24 hr. Radioactivity and unchanged ester were determined in the 24-hr excreta and expired air and in the carcass, and about 91% of the administered carbon-14 was recovered (Table 4). Isotope dilution analysis showed that unchanged ester accounted for the following percentages of administered radioactivity: 0·1% in the tissues analysed (blood, liver and adipose tissue), 1% in the contents of the gastro-intestinal tract, 1% in the urine and 7% in the faeces. These data suggest that, in the 24 hr after treatment, about 9% of the administered substance was recovered in its unchanged form

Table 2. *Identification and determination of unchanged [^{14}C]octyl gallate in the rat liver, by isotope dilution analysis, 10 min after oral administration of [^{14}C]octyl gallate (15 mg/kg; 0·22 mCi/kg)*

No. of recrystallizations	Specific activity of [^{14}C]OG (dpm/mg)
1	14,400
2	14,200
3	5500
4	3800
5	2120
6	1970
7	1960
8	1980
9	2020
10–15	1990 ± 10†

*With addition of 100 mg unlabelled octyl gallate to 3·75 g liver tissue (wet weight).
†Mean ± 1 SD for six recrystallizations.

Table 3. *Content of unchanged [¹⁴C]octyl gallate in liver, adipose tissue and blood of rats treated orally with [¹⁴C]octyl gallate in a dose of 15 mg/kg (0·22 mCi/kg)*

Tissue/fluid	Content of unchanged [¹⁴C]OG at:		
	10 min*	30 min*	6 hr*
Liver—μg/g	1·6	0·05	0·04
—%†	8	1	1
Adipose tissue—μg/g	0·02	0·3	0·05
—%†	8	22	7
Blood—μg/ml	ND	0·1	ND
—%†	—	5	—
Gut wall—μg/g	107	71	ND
—%†	53	38	—

ND = Not determined
*Time after treatment; one rat was killed at each time.
†Radioactivity present as the unchanged ester expressed as a percentage of the total radioactivity in the sample.

and that by far the major portion of the dose was metabolically altered.

Discussion

The fate of octyl gallate in the animal was found to be characterized by limited enteral absorption and rapid biotransformation. Tissue analyses indicated absorption of about 10% of the administered ester, but this figure may be more than twice as high if one considers the high rate of metabolism. The half-life of the ester calculated from the decline in the content of octyl gallate in the liver and in the wall of the gastro-intestinal tract during the 30-min interval after administration is estimated to be less than 10 min. We presume that the ester undergoes hydrolysis, as reported for propyl and lauryl gallate (Booth, Masri, Robbins *et al.* 1959; Dacre, 1960). Support for this hypothesis is seen in the expiration of labelled carbon dioxide by the [¹⁴C]octyl gallate-treated rat. It very probably indicates cleavage of the ester linkage of octyl gallate, followed by biodegradation of octyl alcohol with rapid formation of labelled CO_2 from the C_1-position of the octanol. Additional support for this hypothesis was derived from *in vitro* experiments in which OG was found to be hydrolysed enzymatically by homogenates of liver and of small intestine (Koss, unpublished observations, 1981). It remains an open question, however, whether the micro-organisms in the lumen of the gastro-intestinal tract play a role in the biotransformation of this ester.

On the basis of the results presented in this study, the conclusion can be drawn that the intake of octyl gallate used as a food additive should not give rise to great toxicological concern. There is no doubt that a certain amount of the ester is absorbed from the gut, but its biodegradation, which was found to be a rapid process, very probably leads to the formation of *n*-octyl alcohol and gallic acid. These two substances are constituents of human food (Acker, Bergner, Diemair *et al.* 1965) and may have a low toxicity.

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Table 4. *Recovery of radioactivity in the rat carcass, gut contents, expired air and excreta 24 hr after a single ip dose of 15 mg [¹⁴C]octyl gallate/kg (0·18 mCi/kg)*

Sample	Recovery of radioactivity (% of dose)
Carcass	8·4 ± 0·4
Gut contents	5·3 ± 3·8
Expired air*	27·8 ± 4·8
Urine*	13·6 ± 3·0
Faeces*	36·3 ± 3·9
Total ...	91·4

*Collected over 24 hr.
Values are means ± 1 SD for groups of three rats.

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NITROSATION OF SARCOSINE, PROLINE AND 4-HYDROXYPROLINE BY EXPOSURE TO NITROGEN OXIDES

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Abstract—The nitrosation of amino acids (sarcosine, proline and 4-hydroxyproline) was investigated in model experiments. The compounds were exposed in crystalline form and as aqueous solutions to defined concentrations of nitrogen oxides (in the range of 1–100 ppm) in the atmosphere of a reaction chamber. Nitrosated amino acids were analysed by GC/TEA after silylation. Generally the extent of nitrosation increased with NO_x concentration, reaction time and air humidity. Nitrosation of aqueous amino acid solutions decreased markedly with increasing pH.

Introduction

Many compounds with secondary amino groups are known to form the corresponding *N*-nitroso compounds by interaction with nitrite in acidic solution. While this kind of nitrosation has been investigated extensively with respect to exogenous and endogenous formation of carcinogenic *N*-nitroso compounds, relatively few studies on nitrosation by nitrogen oxides have been reported. The formation of dialkylnitrosamines by reaction of amines and nitrogen oxides in the gas phase was studied under various experimental conditions (Pitts, Grosjean, van Cauwenbergh *et al.* 1978; Rolle, Gellert & Renner, 1978) and the mechanism of nitrosation of amines in solution by gaseous nitrogen oxides has been investigated (Challis & Kyrtopoulos, 1978 & 1979). Solid substances (e.g. amidopyrine) were also found to be easily nitrosated in the presence of nitrogen oxides (Eisenbrand, Spiegelhalder, Kann *et al.* 1979). The nitrosation of pesticides was studied by exposing them as dry powders or as aqueous suspensions to NO_x in a reaction chamber (Janzowski, Klein & Preussmann, 1980). In these model experiments the effects of various parameters on the nitrosation yields of this group of compounds was investigated.

The study described here was performed to obtain more general information on the nitrosation of solid substances with NO_x. Water-soluble amino acids were selected for study because their physico-chemical properties differ markedly from those of the almost water-insoluble pesticides and because this model might, at least to some extent, simulate the situation in foods coming into contact with gaseous nitrosating agents, for example during direct drying (Spiegelhalder, Eisenbrand & Preussmann, 1980). After exposure of the amino acids, sarcosine, proline and 4-hydroxyproline, to NO_x, the samples were analysed for the *N*-nitrosamino acids formed.

Experimental

Materials. Proline, 4-hydroxyproline and sarcosine were obtained from Merck AG, Darmstadt. The corresponding nitrosated amino acids were available in our laboratory; their synthesis and properties have been described elsewhere (Lijinsky, Keefer & Loo, 1970). Solvents were of analytical grade. Extrelut was obtained from Merck AG, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) from Macherey-Nagel Co., Düren, and nitric oxide (NO, 99·8% purity) from Messer Griesheim GmbH, Düsseldorf.

Reaction of amino acids with nitrogen oxides. Dry powdered amino acids (0·1 g) were put in a Petri dish and exposed to nitrogen oxides (1–100 ppm) in a reaction chamber described previously (Janzowski *et al.* 1980), under varying conditions of humidity, time and pH. Before exposure aqueous solutions (0·1 g amino acid/2 ml) were adjusted to the desired pH values with a citrate buffer (0·1 M-sodium citrate–0·1 N-HCl, 4:6 v/v) for pH 3 and with phosphate buffers containing 0·067 M-Na_2HPO_4–0·067 M-KH_2PO_4 in v/v ratios of 1:99 for pH 5, 61:39 for pH 7 and 96:4 for pH 8. At the selected air flow, a ratio of 2:1 (v/v) for NO/NO_2 was established in the reaction chamber and controlled by measuring the actual NO_2 concentration in the chamber using an electrolytic analyser (Interscan, Dallas, TX, USA).

Isolation and determination of N*-nitrosamino acids in the reaction mixture.* A Thermal Energy Analyzer (TEA; Thermo Electron Corp., Waltham, MA, USA) was used for detection of the nitroso products. Dry samples were dissolved in 3 ml 10% sodium ascorbate solution (pH 6), adjusted to pH 1, saturated with sodium chloride and placed on an Extrelut column (length 11 cm, ID 2 cm). After addition of 6 ml sodium ascorbate solution, the column was eluted with 30–40 ml ethyl acetate–methanol (9:1, v/v). A measured aliquot of the organic phase was evapor-

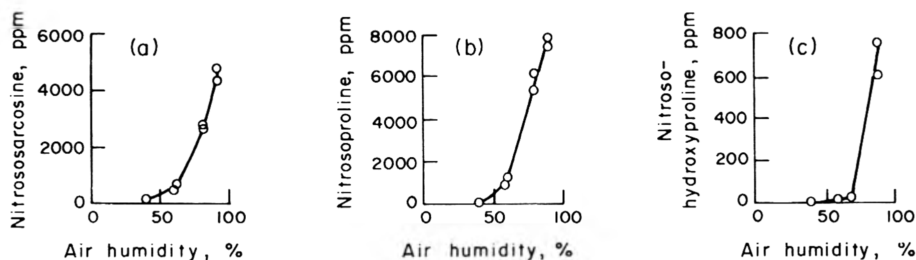

Fig. 1. Effect of air humidity on the nitrosation of (a) sarcosine, (b) proline and (c) 4-hydroxyproline exposed in powdered form to 100 ppm NO_x for 16 hr.

ated to dryness, and then MSTFA (0·2 ml) was added for silylation and allowed to react at room temperature for about 30 min (Eisenbrand, Janzowski & Preussmann, 1975). Trimethylsilyl derivatives of the nitrosamino acids were analysed by gas chromatography using a TEA detector. Separations were carried out on a glass column (1·2 m × 2·0 mm) packed with 6% OV-17 on Gas Chrom Q. The oven temperature was 95°C isothermal for 10 sec, and was then programmed to rise by 10°C/min to 200°C; the gas flow (argon) was 30 ml/min.

Recoveries were in the range of 90–100% for *N*-nitrososarcosine and *N*-nitrosoproline and 50–70% for *N*-nitroso-4-hydroxyproline.

Assessment of grain sizes. Mean grain sizes were estimated roughly by means of a calibrated ocular micrometer according to Batel (1971).

Calculation of results. Nitrosation yields are expressed in parts per million of the theoretical yield calculated on the amine concentration.

Results and Discussion

At constant humidity, the yields of nitrosamino acids from powdered sarcosine, proline and 4-hydroxyproline were found to be directly related to the NO_X concentration. The influence of air humidity on the nitrosation of solid amino acids is shown in Fig. 1. Nitrosation rates were strongly enhanced by an increase in moisture. In contrast, the nitrosation of several poorly water-soluble pesticides investigated earlier was inversely related to air humidity (Janzowski *et al.* 1980). Evidently, the effect of air humidity depends on the physico-chemical properties of the compounds to be nitrosated. In the case of the only slightly water-soluble pesticides a thin layer of water, adsorbed onto the surface of the crystals, impairs gas–solid nitrosation by reducing the direct contact with NO_x. The hygroscopic amino acids, on the other hand, partially dissolve in the absorbed water, and therefore are more easily nitrosated as air moisture increases. In this situation, nitrogen oxides, which also dissolve and hydrolyse in the absorbed water, generate nitrosation conditions similar to those in aqueous acid.

The dependence of the reaction on time was investigated at NO_x concentrations of 100, 20, 10 and 2 ppm. The time course of nitrosations with 100 ppm NO_x is shown in Fig. 2. Solid 4-hydroxyproline was nitrosated to a very small extent, compared with sarcosine and proline, one possible reason being its larger grain size (mean 0·8 mm) compared with that of proline (0·1 mm) and sarcosine (0·25 mm). It may, however, also be speculated that the 4-hydroxy group of 4-hydroxyproline could react preferentially with NO_x, preventing extensive *N*-nitrosation or leading to an *O*-nitroso-*N*-nitroso- derivative, which would escape the analytical determination. The shape of the graphs in Fig. 2 shows that nitrosation of all these amino acids increased overproportionally with respect to the reaction time. In this respect nitrosation of the amino acids differs from that of the previously tested pesticides, which approached saturation after certain times of exposure (Janzowski *et al.* 1980). This difference between the two groups of compounds also suggests a differential effect of surface water. During the time of exposure, hygroscopic amino acids and nitrogen oxides partially dissolve in the absorbed water and react to form *N*-nitrosamino acids. This might favour further nitrosation by decreasing the pH. With pesticides, however, the absorption of water counteracts the nitrosation, as already discussed.

Nitrosation of aqueous amino acid solutions exposed to an atmospheric NO_x concentration of 100 ppm is shown in Fig. 3. In contrast to the nitrosa-

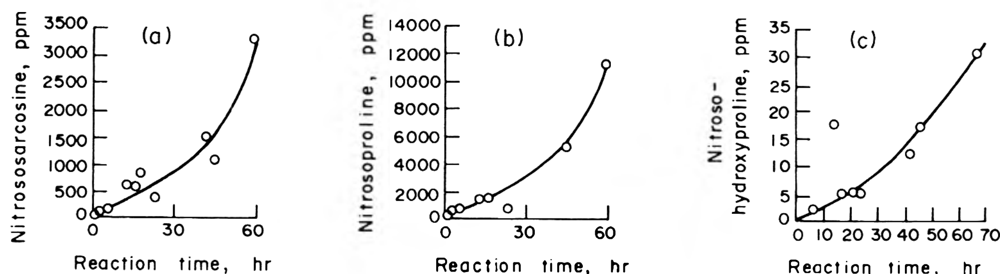

Fig. 2. Effect of reaction time on the nitrosation of (a) sarcosine, (b) proline and (c) 4-hydroxyproline exposed as dry powder to 100 ppm NO_x.

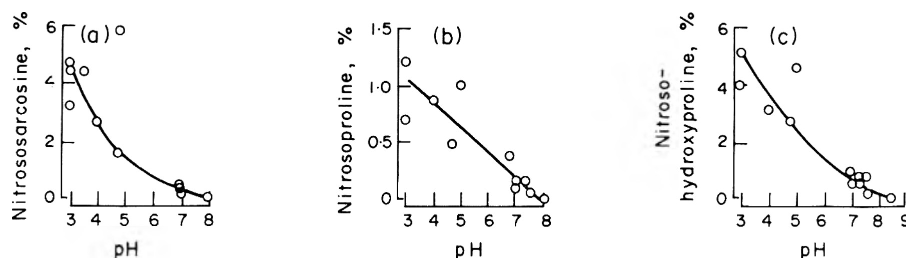

Fig. 3. Effect of pH on the nitrosation of (a) sarcosine, (b) proline and (c) 4-hydroxyproline exposed in aqueous solution to 100 ppm NO_x for 4 hr.

tion of undissclved amino acids, the nitrosation rates obtained in aqueous solution were similar for sarcosine, proline and 4-hydroxyproline. Nitrosation increased markedly with decreasing pH, suggesting that the nitrosation species is nitrous acid rather than nitrogen oxides.

The results of our studies permit a rough estimation to be made of the possible formation of non-volatile *N*-nitroso compounds by NO_x. Exposure to NO_x concentrations of 0·1–1 ppm, which is the range found in the atmosphere (Kellner, 1975), is likely to generate *N*-nitroso compounds in concentrations that are at most a few ppm of the quantity of the amine itself. Under specific conditions, however, higher NO_x concentrations will give rise to higher yields of nitroso derivatives. The possibility should be taken into consideration when food-drying processes utilizing direct-firing techniques are to be used.

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INFLUENCE OF *MYCOPLASMA ARGININI* INFECTION ON THE INDUCTION OF ARYL HYDROCARBON HYDROXYLASE BY TCDD IN RAT HEPATOMA CELL CULTURES

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Abstract—*Mycoplasma arginini* was eliminated from a rat hepatoma cell line (H-4-II-E) by plating at low cell density and treatment with chlortetracycline (250 μg/ml), kanamycin (250 μg/ml), tylosin (100 μg/ml), 3% *M. arginini* antiserum and 5% fresh guinea-pig serum. The induction of AHH activity in the cell culture was measured in response to increasing concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The ED_{50} values (estimated doses that produce 50% maximum enzyme induction) were calculated to be 0·256, 0·452 and 0·344 pmol TCDD/plate for original, mycoplasma-free and reinfected cells, respectively. Although the absence of *M. arginini* in the rat hepatoma cell line makes the cells slightly less responsive to AHH induction by TCDD, this decrease does not detract from the use of the method to screen food extracts and environmental samples for the presence of certain toxic planar organic compounds.

Introduction

Inadvertent infection of mammalian cell cultures by mycoplasmas is a well-known problem which can complicate biochemical and genetic studies. Van Diggelen, McGarrity & Shin (1978) demonstrated that the five most frequently encountered species of mycoplasma, including *Mycoplasma arginini*, contribute significant levels to hypoxanthine phosphoribosyl transferase (HPRT) activity in mouse cell lines. Other biochemical consequences of mycoplasma contamination of cell cultures have been reviewed by Stanbridge (1971). Barile & Kern (1971) and Barile, Hopps, Grabowski *et al.* (1973) reported that the major source of *M. arginini* contamination is bovine serum.

The induction of aryl hydrocarbon hydroxylase (AHH) activity in a rat hepatoma cell line (H-4-II-E) provides a sensitive and reproducible method of detecting picogram amounts of certain toxic planar organic compounds including many dibenzo-*p*-dioxins, dibenzofurans and biphenyls (Bradlaw & Casterline, 1979; Bradlaw, Garthoff, Hurley & Firestone, 1980). This method is useful for screening food extracts that are suspected of containing these toxic substances prior to chemical identification. Response to the most reactive compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is used as a standard curve and AHH activity induced by TCDD is reported as the ED_{50} (estimated dose that produces 50% maximum enzyme induction). The presence of *M. arginini* in this rat hepatoma cell line raised the question of the influence of contaminating mycoplasma on AHH induction.

Rescue of a critical cell line from a mycoplasma-contaminated culture has been attempted by injecting the cells into a nude mouse and culturing cells from the resultant tumour (Van Diggelen, Shin & Phillips, 1977), and by treatment of cell cultures with antibiotics (Buskirk, 1967) or specific antiserum (Pollock & Kenny, 1963) or by combinations of these treatments (Barile, 1979). However, such procedures can induce selection and the cells may not retain the original properties of the culture (Barile, 1979; Pollock & Kenny, 1963).

Knutson & Poland (1980) screened 23 different cell lines for AHH induction in response to TCDD and found that the H-4-II-E-C3 subline V was the only cell line that elicited the desired properties of low basal activity and high AHH activity induced by TCDD. Although increased AHH activity could be induced by TCDD in several of the cell lines, the basal AHH activity was also high and would complicate measurements in a standard curve response. Therefore, for our purposes it was necessary to attempt to decontaminate the cell line rather than to look for a substitute.

In this communication, we describe the successful elimination of *M. arginini* from the H-4-II-E rat hepatoma cell line by the combined treatments of plating at low cell number, applying several antibiotics at the maximum tolerated doses and applying *M. arginini* specific antiserum. In addition, we demonstate that these treated cells retain their ability to respond to TCDD by induction of AHH activity in the standard assay. For the purpose of screening food extracts, however, it may not be important that the cell line is contaminated with mycoplasma, as long as the re-

Abbreviations: AHH = Aryl hydrocarbon hydroxylase; FDA = Food and Drug Administration; HPRT = hypoxanthine phosphoribosyl transferase; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

sponse to the standard chemical remains relatively constant.

Experimental

Cells were maintained in minimal essential medium with Earle's salts (Flow Laboratories, McLean, VA), supplemented with 10% heat-inactivated calf serum, 10% foetal bovine serum (Reheis Biochemicals, Kankakee, IL), 100 units penicillin/ml and 100 μg streptomycin/ml, and were incubated at 37°C in 5% CO_2 in air. A description of the rat hepatoma cell line H-4-II-E, the procedure for the cell culture–AHH induction method and discussions of detection and quantitation limits have been published elsewhere (Bradlaw & Casterline, 1979; Bradlaw *et al.* 1980). The standard TCDD (99·5% pure) stock solution contained 1·6 μg TCDD/ml isooctane and was provided by D. Firestone, Bureau of Foods, Food and Drug Administration (FDA), Washington, DC.

The presence of *M. arginini* in the cell line was detected by routine mycoplasma isolation and species identification procedures at M. A. Bioproducts, Bethesda, MD, and confirmed by R. DelGiudice, Frederick Cancer Research Center, Frederick, MD, using standard methods (Barile, 1974 & 1980; McGarrity, 1975a,b). The procedures to obtain mycoplasma-free cells were suggested by M. Barile (Bureau of Biologics, FDA, Bethesda, MD), who also supplied the *M. arginini* antiserum (lot MB 732-501-071).

Preliminary experiments were performed to determine the maximum tolerated dose of several antibiotic combinations. Cells were seeded in 60 × 15-mm petri plates at concentrations of 100 or 500 cells/5 ml medium. To the medium were added various combinations of chlortetracycline at 100, 250 or 500 μg/ml, kanamycin at 100, 250 or 500 μg/ml, and tylosin at 100 or 200 μg/ml; these antibiotics were purchased from Grand Island Biochemical Co., Grand Island, NY. Colonies were allowed to grow for 7 days, and were then fixed and stained with 2% methylene blue. The combinations of antibiotics at various concentrations that permitted the development of colonies containing 100 or more cells were determined and these dose levels were used in the following experiments.

Cells, beginning with 260 cells/plate, were passaged three times in 5 ml minimal essential medium containing penicillin and streptomycin plus chlortetracycline (250 μg/ml), kanamycin (250 μg/ml), tylosin (100 μg/ml), 3% *M. arginini* antiserum and 5% fresh guinea-pig serum (GIBCO, Grand Island, NY) to provide complement. Two additional passages were made in medium that contained no antiserum but to which the antibiotics were added and a final passage was made in medium without antibiotics. It took 6 wk to carry out this procedure because the cells grew slowly in the antibiotic-supplemented medium. The cells were then frozen and stored at the temperature of liquid nitrogen. An ampule of cells was thawed, reseeded and passaged six times in medium without antibiotics. *M. arginini* was not detected in any of the six cultures or in the frozen cell stock in tests at M. A. Bioproducts. However, the original cell line remained positive for induction of AHH activity by TCDD. This observation was subsequently confirmed at the American Type Culture Collection, Rockville, MD.

Cultures of the original cells and mycoplasma-free cells were grown to confluence in the basic medium. Several of the mycoplasma-free cells were reinfected by the addition of 10% spent medium from cultures of the original cells. After 3 days incubation, confluent cultures of original cells, mycoplasma-free cells and reinfected cells were collected by trypsinization. Replicate samples (0·5 ml) of each culture (10^6 cells) were added to a 2-ml suspension of spent medium plus loose cells from the respective cultures and mycoplasmas were isolated and titrated by standard methods (Barile, 1974 & 1980; McGarrity, 1975a,b) at M. A. Bioproducts.

At the same time, 0·5 × 10^6 cells from each group (contained in 4 ml of basic medium) were added to each of 33 60 × 15-mm plates. After 24 hr incubation, the medium was replaced with fresh medium and various concentrations of TCDD in isooctane were added to each plate (0·1 ml/plate). Three plates were treated per TCDD concentration. Solvent control plates were treated with 0·1 ml isooctane. After 72 hr incubation, the cells were washed with and scraped into phosphate-buffered saline, centrifuged at 500 **g** and stored frozen at −120°C. Samples for enzyme determination (100 μl) and protein analysis (20 μl) were taken after homogenization in Tris-sucrose buffer (pH 8·0). AHH activity was determined by a method described by Nebert & Gelboin (1968) which measures the conversion of benzo[*a*]pyrene to 3-hydroxybenzo[*a*]pyrene. One unit of AHH specific activity is defined as the amount of enzyme catalysing per minute at 37°C the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol of the 3-hydroxybenzo[*a*]pyrene standard. The specific activity was expressed in units/mg cellular protein.

Results and Discussion

M. arginini was identified in both the original and reinfected cells; the results of the mycoplasma titrations are summarized in Table 1. The cells for experiment 1 were passaged from the frozen state four times before their use in the assay procedure and the cells for experiment 2 were passaged only once before they were used. The higher level of mycoplasma in the first group can therefore be explained by the increased time in culture. No mycoplasma infection was detected in the "cured" cells.

The absolute maximum enzyme specific activity in experiments 1 and 2 was 95 and 113 units/mg protein/min, respectively. The response as a fraction of the maximum was used to normalize the data with regard to induction of AHH activity and to provide a uniform comparison between the cell types. The data from the two experiments have been combined and AHH induction (as the mean fractional response) in the three types of cells at increasing doses of TCDD is shown in Table 2. The AHH activity in the solvent-control cells was less than 1·0 unit/mg protein/min, and was often too low to measure accurately. The means were compared pairwise at each concentration using a *t* test technique, and the statistically significant differences are shown in Table 2. AHH activity induced by TCDD in mycoplasma-free cells differed from that in original cells at only the low doses of TCDD ((0·032–0·24 pmol/plate). At 0·32 pmol/plate

Table 1. *Mycoplasma isolation and identification*

Cell sample	Experiment 1			Experiment 2			Species
	Mycoplasma detection		Titration (colony forming units/ 0·2 ml)*	Mycoplasma detection		Titration (colony forming units/ 0·2 ml)	
	Culture on agar	DNA staining		Culture on agar	DNA staining		
Original	+	NT	23 × 10^3	+	NT	10^3	*M. arginini*
Mycoplasma-free	ND	ND	ND	ND	ND	ND	ND
Reinfected	+	NT	10^3	+	NT	10^3	*M. arginini*

NT = Not tested ND = Not detected

*Samples had been frozen and thawed twice when titrations in soft agar were performed.

Tests for mycoplasma contamination were carried out by culture on agar media and, for the mycoplasma-free cell culture, by DNA staining using a fluorescent bisbenzimidazole compound (Hoechst 33258). Titration was carried out by standard methods. For further details see Barile (1974 & 1980) and McGarrity (1975a,b).

and above, there were no significant differences between the two cell types. AHH activity induced by TCDD in reinfected cells differed from that in the original cells at TCDD levels of 0·128, 0·16 and 0·24 pmol/plate, and from activity in the mycoplasma-free cells at 0·08, 0·128 and 0·16 pmol/plate. The ED_{50} values were determined by probit analysis of data for dose levels between 0·032 and 3·2 pmol TCDD/plate. The ED_{50} values for the original cells, mycoplasma-free cells and reinfected cells were 0·256, 0·452 and 0·344 pmol/plate, respectively. The absence of *M. arginini* in the culture makes the cells slightly less responsive to AHH induction by TCDD and places the limit of detection at 0·08 pmol TCDD/plate (25 pg TCDD) in comparison with 0·032 pmol/plate (10 pg TCDD) determined for the original cells (Bradlaw & Casterline, 1979).

The contribution of *M. arginini* to the AHH activity associated with the cellular response to TCDD is not evident from this study, as the observed differences in AHH activity could also be a consequence of the antibiotic treatment regime. The low titre of *M. arginini* in the reinfected cell sample does not allow a direct comparison with the original cell sample in terms of number of mycoplasma. If strains of *M. arginini* express HPRT activity (Van Diggelen *et al.* 1978), they may also express basal and inducible AHH activity. The most encouraging findings were the successful elimination of *M. arginini* from the cells and the ability to induce with TCDD a maximum enzyme activity in the mycoplasma-free cells that was equivalent to that of the original cells.

Quality control practices are the best means of avoiding mycoplasma contamination and should be used at all times (McGarrity, 1975a,b & 1976; McGarrity & Coriell, 1971). Our objective of eliminating *M. arginini* from the rat hepatoma cell line H-4-II-E was achieved, in part, because the species of mycoplasma had been identified and the specific antiserum to *M. arginini* was available. In addition, *M.*

Table 2. *Mean fractional responses of AHH induction for original cells, mycoplasma-free cells and reinfected cells from two experiments*

TCDD (pmol/plate)	Fractional response of AHH induction in Original cells	Mycoplasma-free cells	Reinfected cells
0 (solvent control)	0·015 ± 0·010	0·017 ± 0·004	0·013 ± 0·006
0·032	0·059 ± 0·017	0·017 ± 0·005*	0·036 ± 0·011
0·08	0·192 ± 0·024	0·065 ± 0·011*	0·170 ± 0·013†
0·128	0·342 ± 0·031	0·162 ± 0·019*	0·240 ± 0·030*†
0·16	0·430 ± 0·055	0·173 ± 0·008*	0·276 ± 0·024*†
0·24	0·546 ± 0·033	0·331 ± 0·023*	0·360 ± 0·039*†
0·32	0·551 ± 0·044	0·476 ± 0·033	0·527 ± 0·031
0·64	0·751 ± 0·053	0·642 ± 0·066	0·677 ± 0·029
1·6	0·839 ± 0·022	0·819 ± 0·029	0·922 ± 0·045
3·2	0·977 ± 0·071	0·948 ± 0·034	0·991 ± 0·064
32·2	0·995 ± 0·064	1·020 ± 0·040	0·917 ± 0·018
ED_{50}‡ ...	0·256 (0·297, 0·223)	0·452 (0·521, 0·397)	0·344 (0·401, 0·299)

AHH = Aryl hydrocarbon hydroxylase TCDD = 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

‡The upper and lower fiducial limits are given in brackets. ED_{50} values and the fiducial limits were derived by probit analysis of the data for the dose levels between 0·032 and 3·2 pmol TCDD/plate.

Values are means ± SEM for three plates/experiment from two experiments. Those marked with an asterisk or dagger are significantly different (*t* test) from the values for the original cells (*$P < 0·05$) or the mycoplasma-free cells (†$P < 0·05$), respectively.

arginini is found predominantly in the extracellular environment bound to the cell membrane (Stanbridge, 1971). Our treatment regime would probably not have been successful for a species of mycoplasma that resides intracellularly.

The absence of *M. arginini* in the rat hepatoma cell line makes these cells less responsive to AHH induction by TCDD, since it is necessary to approximately double the TCDD dose to achieve half-maximal enzyme induction. However, this decrease in no way detracts from the effectiveness of the method as a screen for the presence of certain toxic organic compounds, such as polyhalogenated biphenyls, dibenzo-*p*-dioxins and dibenzofurans in food extracts and environmental samples. TCDD is at least tenfold more potent than other chemicals tested to date. The advantages of distributing a mycoplasma-free cell line to laboratories who wish to use the method may outweigh the disadvantage of a slight decrease in sensitivity of the method. These cells are available and distributed through the American Type Culture Collection as ATCC.CRL-1548.

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Review Section

REPEATABILITY AND REPRODUCIBILITY OF DETERMINATIONS OF VINYL CHLORIDE IN FOODSTUFFS

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Abstract—In the spring of 1978 and the autumn of 1979, two collaborative investigations were carried out with the aim of determining the variability of measurements of the vinyl chloride (VC) content of foodstuffs. The first investigation was a preliminary test on VC standards in *N,N*-dimethylacetamide. The results of this test were considered to be satisfactory. In a second test, lemonade and oil samples spiked with VC were investigated. The methods used were based on the headspace gas-chromatographic technique. The amount of VC added was 3 μg/kg for the lemonade sample and 20 μg/kg for the oil sample. The external standard method yielded a reproducibility of 53 and 39% and a repeatability of 18 and 17% for the lemonade and oil, respectively. The corresponding findings with the addition method were a reproducibility of 119 and 40% and repeatability of 33 and 9%.

Introduction

On 30 January 1978, the Council of the European Communities (1978) adopted a Directive setting at 1 mg/kg the maximum vinyl chloride monomer level for materials and articles of PVC. In order to establish the analytical methodology necessary to check compliance with this limit and in order to determine the variability of the method, two collaborative studies were carried out in a number of experienced laboratories under the supervision of the Commission of the European Communities. The results of these two collaborative studies were described in a previous publication (Rossi, Waibel & vom Bruck, 1980).

However, the 1978 Directive was not concerned solely with levels of residual monomer in the PVC materials. Article 2.2 of the Directive stated that "Materials and articles must not pass on to foodstuffs which are in or have been brought into contact with such materials and articles any vinyl chloride detectable by the method which complies with the criteria laid down in Annex II", those criteria being the use of the headspace gas-chromatographic technique and a detection limit of 0·01 mg VC/kg foodstuff. In the light of this requirement, the need arose to carry out at Community level other collaborative studies to establish a method of analysis for determining VC in foodstuffs and to check the variability of this method.

*Chairman of the Commission of the European Communities Working Party on "Materials and articles intended to come into contact with foodstuffs".

Abbreviations: DMA = *N,N*-Dimethylacetamide; GC = gas chromatography; IS = internal standard; PVC = polyvinyl chloride; VC = vinyl chloride monomer.

The variability is expressed as repeatability (r) and as reproducibility (R), defined as follows in accordance with ISO/DIS 5725 (International Organisation for Standardisation, 1977):

Reproducibility is "the value below which the absolute difference between two single test results obtained with the same method on identical test material, under the same conditions (same operator, same apparatus, same laboratory, and a short interval of time), may be expected to lie with a specified probability; in the absence of other indications the probability is 95%".

Reproducibility is "the value below which the absolute difference between two single test results obtained with the same method on identical test material, under different conditions (different operators, different apparatus, different laboratories and/or different time), may be expected to lie with a specified probability; in the absence of other indications the probability is 95%".

This paper reports the results of two collaborative studies and includes a description of the analytical method used.

First EEC collaborative study (1978)

In the spring of 1978 the Commission of the European Communities, in collaboration with the Food Inspection Service (Utrecht, The Netherlands), organized a collaborative study in which 16 of the EEC laboratories listed in Appendix I participated.

The study involved determining unknown levels of VC in *N,N*-dimethylacetamide (DMA) solutions. Each laboratory was required to send its own empty

Table 1. *Results of first (1978) collaborative study on the determination of vinyl chloride in dimethylacetamide solutions*

Sample	Mean VC concn estimated (mg/kg)*	Repeatability (mg/kg)	Reproducibility (mg/kg)	No. of participants
A	0·052	0·005	0·009	12
B	0·138	0·010	0·017	13

*These mean figures coincided with the concentrations of the solutions as prepared.

headspace vials to Utrecht, where they were filled with VC solutions in DMA (0·05–0·20 mg VC/kg). The exact concentrations were not known to the analysts. To meet the requirements of the participants intending to use an internal standard (IS), diethyl ether was added in a stated concentration of 1·963 mg/kg. Preparation of samples was carried out according to the method described in Appendix II (5.1). The vials were sealed and sent to the laboratories, precautions being taken to avoid the loss of VC, through volatilization, during dispatch.

The vials were then used directly for the determination carried out according to the external standard (calibration) method described in principle by Rossi *et al.* (1980). The liquid sample of VC in DMA was allowed to reach equilibrium with the vapour phase, which was then sampled by the GC headspace technique for the VC determination. The method is not exhaustively described and therefore the participants were free to choose the GC column, the amount of sample, the eventual IS and the type of apparatus (automatic or manual) for sample injection.

Two samples of each concentration were prepared and designated A_1 and A_2 and B_1 and B_2. It was not revealed to the participants that A_1 and A_2 (and similarly B_1 and B_2) contained the same concentration of VC. Results were calculated by every laboratory and the statistical evaluation was carried out in Utrecht according to ISO/DIS 5725. The results are summarized in Table 1. The actual concentrations of VC were 0·052 and 0·138 mg/kg in the A and B samples, respectively. These values were the same as the means of the participants' findings. The means of the values for variability obtained at the two test levels were 8 and 15% of the actual concentration for repeatability and reproducibility, respectively. These results were considered very satisfactory by all the participants, taking into account the fact that not all the details of the method were standardized.

Second EEC collaborative study (1979)

The second EEC collaborative study was intended to ascertain the variability of the measurements of VC in two major foodstuffs as well as the differences in the repeatability and reproducibility values obtained with the external standard method (Rossi *et al.* 1980) and with the addition method (see Appendix II).

Preparation of the samples

Standard solution of VC in DMA. To 96·9368 g DMA was added 0·9182 g VC, and then 0·6894 g of this solution was diluted by addition of 96·7895 g DMA.

Lemonade. To 1 litre of lemonade, made from orange lemonade syrup (van Welie, Zeist) by dilution with tap-water, was added 0·9570 g of the standard solution of VC in DMA. This was then diluted in an appropriate flask to 10 litres to give a VC concentration of 6·46 μg/litre.

Salad oil. To 1 litre of salad oil (a mixture of vegetable oils, such as arachis oil and soya oil, supplied by Remia Holland, Den Dolder, The Netherlands) was added 2·9035 g of the standard solution of VC in DMA. This was then diluted in an appropriate flask to 10 litres to give a VC concentration of 19·59 μg/litre.

Distribution of test solutions. Using a siphon, 250-ml flasks were filled from these 10-litre flasks and were immediately closed with a rubber septum with teflon insert and an aluminium screw-cap. The filling sequence corresponded to the participant number.

Results and statistical analysis

External standard method. The statistical evaluation of the results was carried out before the Directive (Commission of the European Communities, 1981; see Appendix II) was available. In contrast to section 5.1.3 of the Directive, the response curve was calculated by means of linear regression *through* the origin. If the response curve had not been forced through the origin the repeatabilities and reproducibilities would be slightly smaller than those reported here.

Tables 2 and 3 present the individual values for the VC concentrations in the two samples, calculated by means of the estimated response curve. Because for any one participant the concentrations were estimated using the same calibration line, they will not be uncorrelated. However, it can be shown that the correlation between measurements by the same participant can be neglected. The statistical evaluation of the results was made according to ISO/DIS 5725.

Tables 2 and 3 show that some participants supplied doubtful results. In some of these cases an explanation was offered by the participant, while in others, the cause (such as the wrong sample or changes in peak heights) was found by studying the submitted sheets, which described details of the procedure. For these reasons, the results of the following participants were excluded from the calculations: for the salad oil determinations, participants 19 (with and without IS) and 32 (Table 2); for the lemonade determinations, participants 19 (with and without IS), 26 and 32 (Table 3). Then the repeatability and reproducibility were calculated according to ISO/DIS 5725 (Table 4).

On the basis of statistical tests for outliers (International Organisation for Standardisation, 1977), the

Table 2. *Results of the collaborative study on the determination of vinyl chloride in salad oil, according to the external standard method*

Participant no.	Concn of VC in sample (μg/kg)					
	Individual determinations					Mean
1	20·6	20·6	—	—	—	20·6
7	23·2	23·0	23·6	22·0	21·0	22·8
7 (IS)	21·2	21·1	20·2	20·3	19·5	20·5
8	21·3	21·0	21·8	22·0	21·8	21·6
9	22·2	23·0	23·5	17·0*	—	21·4
9 (IS)	24·2	24·0	23·5	22·7	—	23·6
11	21·3	21·3	21·3	—	—	21·3
14	20·0	19·6	19·6	20·0	20·5	19·9
16	14·7	15·3	16·6	19·0	19·0	16·9
16 (IS)	14·1	15·4	17·5	17·6	18·1	16·6
19†	29·2	41·2	41·4	—	—	37·3
19† (IS)	29·5	46·0	60·2	—	—	45·2
20	16·4	19·3	19·3	16·4	19·3	18·1
21	27·7	22·6	21·9	20·4	21·7	21·7
22	19·1	21·4	20·9	21·4	20·6	20·7
25	24·1	20·8	20·6	23·7	21·0	22·0
26	18·2	18·2	16·1	13·9	16·1	16·5
29	16·4	15·8	16·0	15·4	16·3	16·0
30	19·2	17·1	15·1	17·0	15·4	16·7
31	22·3	22·5	22·5	21·8	22·5	22·3
32†	15·2	28·3	61·7	62·7	70·4	47·6

IS = Determinations with internal standard
*Result discarded prior to calculation of repeatability and reproducibility.
†All three or five determinations discarded prior to calculation of repeatability and reproducibility.

Table 3. *Results of the collaborative study on the determination of vinyl chloride in lemonade, according to the external standard method*

Participant no.	Concn of VC in sample (μg/kg)					
	Individual determinations					Mean
1	2·4	2·7	—	—	—	2·5
7	3·7	4·1	3·4	3·7	3·7	3·7
7 (IS)	2·5	2·8	2·5	2·6	2·5	2·6
8	3·8	3·8	3·8	3·7	3·9	3·8
9	4·3	3·6	3·6	3·7	4·2	3·9
9 (IS)	3·4	3·5	3·5	3·5	4·2	3·6
10	3·2	3·6	3·6	4·1	4·1	3·7
11	4·6	5·1	4·9	—	—	4·9
14	2·1	2·1	2·0	1·9	2·1	2·0
16	3·2	3·4	3·1	3·2	3·0	3·2
16 (IS)	3·2	3·2	3·1	2·9	2·9	3·0
18*	3·2	3·2	4·2	4·2	3·2	3·6
19*	25·4	38·5	53·0	70·4	—	46·8
19* (IS)	29·0	42·4	56·2	76·2	—	51·0
20	3·7	4·0	3·4	3·7	3·7	3·7
21	3·4	3·6	3·3	3·7	3·1	3·4
22	3·3	—	3·3	3·0	3·2	3·2
25	4·0	4·0	3·9	3·8	4·5	4·1
26*	0·2	0·2	0·5	0·8	0·8	0·5
29	3·2	3·2	2·4	2·4	3·3	2·9
30	3·1	3·0	3·0	3·2	3·3	3·1
31	4·0	3·9	4·0	3·9	3·9	4·0
32*	14·2	23·9	31·6	36·1	50·9	31·3

IS = Determinations with internal standard
*All four or five determinations discarded prior to calculation of repeatability and reproducibility.

fourth measurement of participant 9 in Table 2 (Dixon's test) and all measurements of participants 18 and 26 in Table 3 (Cochran's test) were excluded. When calculations were made with and without outliers, there was almost no difference in the relevant statistics. We report here only the results without outliers.

Calculations for the repeatability and reproducibility where an IS was used are based on the results of only a few participants and should be interpreted with caution.

Addition method. Tables 5 and 6 summarize the results obtained by application of the addition method. Applying the same procedure as was used in the calculation of the results by the external method, some doubtful results were excluded, namely the results of participants 7, 10, 19 (with and without IS), 30 and 32 in Table 5 and those of participants 19 (with and without IS), 26 and 32 in Table 6.

After rejection of these results, those of participants 9 and 11 in Table 5 as well as the results of participants 7, 9 and 11 in Table 6 became "statistical outliers" according to Cochran's test. After that the repeatability and reproducibility were calculated (Table 7).

The repeatabilities calculated without outliers were much better than those calculated with outliers, 9 and 27% respectively, for salad oil and 33 and 94% for lemonade (all results without IS and expressed as a percentage of the estimated concentration). Calcu-

Table 4. *Statistical evaluation of results of the second collaborative study (external standard method)*

Sample	No. of laboratories		Estimated VC content (μg/kg)	Repeatability (μg/kg*)	Reproducibility (μg/kg*)
	Included	Rejected			
Without internal standard					
Oil	14	3	19·86	3·31 (17)	7·71 (39)
Lemonade	14	5	3·50	0·62 (18)	1·85 (53)
With internal standard					
Oil	3	1	19·16	3·31 (17)	10·30 (54)
Lemonade	3	1	3·09	0·63 (20)	1·57 (51)

*Values in brackets are the repeatability or reproducibility expressed as a percentage of the estimated VC content of the sample.

Table 5. *Results of the collaborative study on the determination of vinyl chloride in salad oil using the addition method*

Participant no.	Concn of VC in sample (μg kg)		
	Individual determinations		Mean
1	20·2	21·4	20·8
7*	35·4	44·3	39·8
7 (IS)	22·2	24·2	23·2
8	20·7	20·7	20·7
9*	19·6	12·7	16·2
9 (IS)	21·2	20·6	20·9
10*	66·8	36·1	51·4
11*	12·5	18·6	15·6
14	20·4	20·1	20·2
16	20·2	19·2	19·7
16 (IS)	16·0	20·5	18·2
17	21·9	21·3	21·6
17 (IS)	22·8	22·6	22·7
19*	30·6	29·3	30·0
19 (IS)*	20·7	16·0	18·4
20	17·3	16·3	16·8
21	21·9	23·7	22·8
22	25·0	23·7	24·4
25	15·3	15·3	15·3
26	18·0	18·0	18·0
29	17·6	17·6	17·6
30*	3·9	0·3	1·8
31	20·3	20·9	20·6
32*	9·7	14·2	12·0

IS = Determinations with internal standard
*Both determinations discarded prior to calculation of repeatability and reproducibility.

Table 6. *Results of the collaborative study on the determination of vinyl chloride in lemonade using the addition method*

Participant no.	Concn of VC in sample (μg kg)		
	Individual determinations		Mean
1	2·9	3·2	3·0
7*	6·8	1·7	4·2
7 (IS)	1·4	3·2	2·3
8	3·3	3·5	3·4
9*	1·8	4·1	3·0
9 (IS)	3·8	3·8	3·8
10	2·9	2·4	2·6
11*	0·7	1·6	1·2
14	4·8	4·1	4·4
16	3·2	3·4	3·3
16 (IS)	3·3	2·6	3·0
17	3·4	4·4	3·9
17 (IS)	3·3	3·9	3·6
19*	8·5	8·6	8·6
19 (IS)*	1·4	1·3	1·4
20	3·2	2·8	3·0
21	3·8	3·8	3·8
22	4·4	4·5	4·4
25	3·6	6·9	5·2
26*	−0·3	−0·4	−0·4
29	6·4	5·7	6·0
30	0·3	1·0	0·6
31	4·0	3·5	3·8
32*	14·1	13·5	13·8

IS = Determinations with internal standard
*Both determinations discarded prior to calculation of repeatability and reproducibility.

lations for the reproducibility when an IS was used are based on the results of only a few participants and should be interpreted with caution.

Conclusions

Table 7 shows that the mean VC concentrations found in oil (19·81–21·26 μg/kg) differ little from the quantity added (19·59 μg/litre). In contrast, the values found in lemonade (3·09–3·50 μg/kg) represented about half of the added amount (6·46 μg/litre). The difference may be tentatively attributed, in the absence of other explanations, to a higher volatility of VC in lemonade than in oil. The repeatability and reproducibility of the results are fairly satisfactory, considering that the method is not standardized in all its details for practical reasons and that the amounts of VC determined are extremely low, i.e. close to the detection limit.

As already demonstrated in the collaborative study on the determination of VC in PVC (Rossi *et al.* 1980), comparison of Tables 4 and 7 shows that the more reproducible results are obtained with the external standard (calibration) method. However at Community level, the addition method, reported fully in Annex II, has been chosen as the official method, because foodstuff blanks (a blank being the same foodstuff not packed in PVC) are often not available, especially in the case of imported products.

Table 7. *Statistical evaluation of results of the second collaborative study (addition method)*

Sample	No. of laboratories		Estimated VC content (μg kg)	Repeatability (μg kg*)	Reproducibility (μg kg*)
	Included	Rejected			
Without internal standard					
Oil	12	7	19·81	1·86 (9)	7·95 (40)
Lemonade	13	6	3·36	1·12 (33)	4·00 (119)
With internal standard					
Oil	4	1	21·26	4·93 (23)	7·26 (34)
Lemonade	4	1	3·17	1·00 (32)	1·00 (31)

*Values in brackets are the repeatability or reproducibility expressed as a percentage of the estimated VC content of the sample.

Table 8. *GLC columns found satisfactory in the collaborative test*

Column						
Material	Length (m)	Diameter (mm)	Stationary phase and loading	Support mesh*	Oven temp. (°C)	Detector
Glass	6·0	2·0	15% SE-30	Chromosorb WHP (60–80)	25	FID
Glass	1·8	2·0		Chromosorb 101 (80–100)	30 and 70	MS
SS	4·0	2·0	15% Ukon LB 550x	Chromosorb WHP (100–120)	60	FID
SS	3 + 3	2·0	3 m 5% Polypropylene glycol 3 m 5% Polyglycol B11/700	Chromosorb (80–100)	40	FID
SS	6·0	3·2	10% Ukon LB 500x	Chromosorb	50	FID
SS	1·5	2·0	0·2% Carbowax 1500	Carbopack C (80–100)	50	MS

SS = Stainless steel FID = Flame ionization detector MS = Mass spectrometer with multiple ion detection

*Figures in brackets indicate mesh size.

The very few data obtained using an IS do not permit any comparison between the results obtained with and without such use. Table 8 lists the columns and oven temperatures used successfully by the participants in the study. It is notable that the two participants using mass spectrometry as a detection method utilized shorter columns than were used by the other participants.

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APPENDIX I

List of institutions and companies participating in the tests

A/S Haustrups Fabriker, Odense, Denmark
BP Chemicals Ltd, Glamorgan, UK
British Industrial Plastics Ltd, Durham, UK
Cadbury-Typhoo Ltd, Birmingham, UK
Centraal Instituut voor Voedingsonderzoek, Zeist, The Netherlands
Chem. Landesuntersuchungsamt, Nordrhein-Westfalen, Münster, Germany
Chem. und Lebensmittelchem. Untersuchungsanstalt Hamburg, Hamburg, Germany
Commissie EEG, Petten, The Netherlands
County Public Health Laboratory, Institute of Preventive Medicine, Glamorgan, UK
DSM Lokatie Kunststoffen, Beek, The Netherlands
Hoechst AG, Gendorf, Germany
ICI Plastics Division, Welwyn Garden City, UK
Ijdsk Teknologisk Institut, Århus C, Denmark
Institut für Lebensmitteltechnologie und Verpackung e.V., München, Germany
Istituto Superiore di Sanità, Roma, Italy
Keuringsdienst van Waren, Utrecht, The Netherlands (two separate laboratories)
Laboratoire Central d'Analyses et de Recherches, Ministère de l'Agriculture, Massy, France
Laboratory of the Government Chemist, London, UK
Metal Box Ltd, UK
Ministerie van Volksgezondheid en van het Gezin, Instituut voor Hygiëne en Epidemiologie, Bruxelles, Belgium
Ministry of Agriculture, Fisheries and Food, Food Laboratories, Norwich, UK
Montedison, Milano, Italy
National Food Institute, Soeborg, Denmark
Odense Kommunes Laboratorium, Odense, Denmark
Reckitt & Colman Ltd, Norwich, UK
Shell Nederland Chemie BV, Rotterdam, The Netherlands
Solvay et Cie, Bruxelles, Belgium
Unilever Forschungsgesellschaft mbH, Hamburg, Germany

APPENDIX II

Determination of vinyl chloride released by materials and articles into foodstuffs*

1. *Scope and field of application*
The method determines the level of vinyl chloride in foodstuffs.
2. *Principle*
The level of vinyl chloride (VC) in foodstuffs is determined by means of gas-chromatography using the 'headspace' method.
3. *Reagents*
3.1 Vinyl chloride (VC), of purity greater than 99·5% (v/v).
3.2 *N,N*-Dimethylacetamide (DMA), free from any impurity with the same retention time as VC or as the internal standard (3.3) under the conditions of the test.
3.3 Diethyl ether or *cis*-2-butene, in DMA (3.2) as the internal standard solution. These internal standards must not contain any impurity with the same retention time as VC, under the conditions of the test.
3.4 Distilled water or demineralized water of equivalent purity.
4. *Apparatus*
N.B. An instrument or piece of apparatus is mentioned only if it is special, or made to particular specifications. Usual laboratory apparatus is assumed to be available.
4.1 Gas-chromatograph fitted with automatic headspace sampler or with facilities for manual sample injection.
4.2 Flame ionization detector or other detectors mentioned in point 7.
4.3 Gas-chromatographic column.
The column must permit the separation of the peaks of air, of VC and of the internal standard, if used.
Furthermore, the combined 4.2 and 4.3 system must allow the signal obtained with a solution containing 0·005 mg VC/litre DMA or 0·005 mg VC/kg DMA to be equal to at least five times the background noise.
4.4 Sample phials of flasks fitted with silicon or butyl rubber septa. When using manual sampling techniques, the taking of a sample from the headspace with a syringe may cause a partial vacuum to form inside the phial or flask. Hence, for manual techniques where the phials are not pressurized before the sample is taken, the use of large phials is recommended.
4.5 Micro-syringes.
4.6 Gas-tight syringes for manual headspace sampling.
4.7 Analytical balance accurate to 0·1 mg.
5. *Procedure*
CAUTION: VC is a hazardous substance and a gas at ambient temperature; therefore, the preparation of solutions should be carried out in a well-ventilated fume cupboard.
N.B.—Take all the necessary precautions to ensure that no VC or DMA is lost;
—When employing manual sampling techniques, an internal standard (3.3) should be used;
—When using an internal standard, the same solution must be utilised throughout the procedure.
5.1 *Preparation of standard VC solution (solution A)*
5.1.1 *Concentrated standard VC solution at approximately 2000 mg/kg*
Accurately weigh to the nearest 0·1 mg a suitable glass vessel and place in it a quantity (e.g. 50 ml) of DMA (3.2). Reweigh. Add to the DMA a quantity (e.g. 0·1 g) of VC (3.1) in liquid or gas form, injecting it slowly onto the DMA. The VC may also be added by bubbling it into the DMA, provided that a device is used which will prevent loss of DMA. Reweigh to the nearest 0·1 mg. Wait 2 hours to allow equilibrium to be attained. If an internal standard is to be employed, add internal standard so that the concentration of the internal standard in the concentrated standard VC solution is the same as in the internal standard solution prepared under point 3.3. Keep the standard solution in a refrigerator.
5.1.2 *Preparation of dilute standard VC solution*
Take a weighed amount of concentrated standard solution of VC (5.1.1) and dilute, to a known volume or a known weight, with DMA (3.2) or with internal standard solution (3.3). The concentration of the resultant dilute standard solution (solution A) is expressed as mg/litre or mg/kg respectively.
5.1.3 *Preparation of the response curve with solution A*

N.B.—the curve must comprise at least seven pairs of points;
—the repeatability of the responses† must be lower than 0·002 mg VC/litre or kg of DMA;
—the curve must be calculated from these points by the least squares technique, i.e. the regression line must be calculated using the following equation:

$$y = a_1x + a_0$$

where
$$a_1 = \frac{n\Sigma xy - (\Sigma x).(\Sigma y)}{n\Sigma x^2 - (\Sigma x)^2}$$

and
$$a_0 = \frac{(\Sigma y)(\Sigma x^2) - (\Sigma x)(\Sigma xy)}{n\Sigma x^2 - (\Sigma x)^2}$$

where

y = the height or area of peaks in any single determination;
x = the corresponding concentration on the regression line;
n = number of determinations carried out ($n \geqslant 14$);

—the curve must be linear, i.e. the standard deviation(s) of the differences between the measured responses (y_i) and the corresponding value of the responses calculated from the regression line (z_i) divided by the mean value ($\bar{y}$) of all the measured responses shall not exceed 0·07.
This shall be calculated from:

$$\frac{s}{\bar{y}} \leqslant 0{\cdot}07$$

where
$$s = \sqrt{\frac{\sum_{i=1}^{n}(y_i - z_i)^2}{n-1}}$$

and
$$\bar{y} = \frac{1}{n}\sum_{i=1}^{n} y_i$$

where

y_i = each individual measured response;
z_i = the corresponding value of the response (y_i) on the calculated regression line;
$n = \geqslant 14$;

*Annex to Commission Directive of 29 April 1981 (81/432/EEC; *Off. J. Europ. Commun.* 1981, **24** (L167), 6).
†See recommendation ISO DIS 5725: 1977.

Prepare two series of at least seven phials (4.4). Add to each phial volumes of dilute standard VC solution (5.1.2) and DMA (3.2) or internal standard solution in DMA (3.3) such that the final VC concentration of the duplicate solutions will be approximately equal to 0, 0·005, 0·010, 0·020, 0·030, 0·040, 0·050, etc. mg/litre or mg/kg of DMA and that each phial contains the same total volume of solution. The quantity of dilute standard VC solution (5.1.2) must be such that the ratio between the total volume (μl) of added VC solution and quantity (g or ml) of DMA, or internal standard solution (3.3) does not exceed 5. Seal the phials and proceed as described under points 5.4.2, 5.4.3 and 5.4.5. Construct a graph in which the ordinate values show the areas (or heights) of the VC peaks of the duplicate solutions, or the ratio of these areas (or heights) to those of the relevant internal standard peaks, and the abscissa values show the VC concentrations of the duplicate solutions.

5.2 *Validation of preparation of standard solutions obtained in point 5.1*

5.2.1 *Preparation of a second standard VC solution (solution B)*

Repeat the procedure described under points 5.1.1 and 5.1.2 to obtain a second dilute standard solution with, in this case, a concentration approximately equal to 0·02 mg VC/litre or 0·02 mg VC/kg of DMA or internal standard solution. Add this solution to two phials (4.4). Seal the phials and proceed as described under points 5.4.2, 5.4.3 and 5.4.5.

5.2.2 *Validation of solution A*

If the average of two gas-chromatographic determinations relating to the solution B (see under point 5.2.1) does not differ by more than 5% from the corresponding point of the response curve obtained in point 5.1.3, the solution A is validated. If the difference is greater than 5%, reject all the solutions obtained in points 5.1 and 5.2 and repeat the procedure from the beginning.

5.3 *Preparation of the 'addition' curve*

N.B.—the curve must comprise at least seven pairs of points;

—the curve must be calculated from these points by the least squares technique (see point 5.1.3, third indent);

—the curve must be linear, i.e. the standard deviation (s) of the differences between the measured responses (y_i) and the corresponding value of the responses calculated from the regression line (z_i) divided by the mean value ($\bar{y}$) of all the measured responses shall not exceed 0·07 (see point 5.1.3, fourth indent).

5.3.1 *Preparation of sample*

The sample of foodstuff to be analysed must be representative of the foodstuff presented for analysis. The foodstuff must, therefore, be mixed, or reduced to small pieces and mixed, before the sample is taken.

5.3.2 *Procedure*

Prepare two series of at least seven phials (4.4). Add to each phial a quantity, not less than 5 g, of sample obtained from the foodstuff under investigation (see point 5.3.1). Ensure that an equal quantity is added to each phial. Close the phial immediately. Add to each phial for each gram of sample 1 ml of distilled water (preferably) or demineralized water of at least equivalent purity, or an appropriate solvent if necessary. (Note: for homogeneous foodstuffs, addition of distilled or demineralized water is not necessary.) Add to each phial volumes of dilute standard VC solution (5.1.2), containing the internal standard (3.3), if considered useful, such that concentrations of VC added to the phials equal to 0, 0·005, 0·010, 0·020, 0·030, 0·040 and 0·050, etc., mg/kg of foodstuffs. Ensure that the total volume of DMA or DMA containing internal standard (3.3) in each phial is the same. The quantity of dilute standard VC solution (5.1.2) and additional DMA where used, must be such that the ratio between the total volume (μl) of these solutions and the quantity (g) of foodstuff contained in the phial is as low as possible but not more than 5 and is the same in all phials. Seal the phials and proceed as described under point 5.4.

5.4 *Gas-chromatographic determinations*

5.4.1 Agitate the phials avoiding contact between the contained liquid and the septum (4.4) to obtain a solution or a suspension of the samples of foodstuff as homogeneous as possible.

5.4.2 Put all the sealed phials (5.2 and 5.3) in a waterbath for 2 hours at 60 ± 1°C to allow equilibrium to be attained. Agitate again, if necessary.

5.4.3 Take a sample from the headspace in the phial. When utilizing manual sampling techniques care must be exercised in obtaining a reproducible sample (4.4), in particular the syringe must be pre-warmed to the temperature of the sample. Measure the area (or height) of the peaks relating to the VC and internal standard, if used.

5.4.4 Construct a graph in which the ordinate value shows the areas (or heights) of the VC peaks or the ratio of the areas (or heights) of VC peaks to the areas (or heights) of the internal standard peaks and the abscissa values show the quantities of VC added (mg) relating to the quantities of the sample of foodstuff weighed in each phial (kg). Measure the abscissa intercept from the graph. The value so obtained is the concentration of VC in the sample of the foodstuff under investigation.

5.4.5 Remove from the column (4.3) excess DMA using an appropriate method as soon as peaks of DMA appear on the chromatogram.

6. *Results*

The VC released by materials and articles into the foodstuff under investigation expressed in mg/kg shall be defined as the average of the two determinations (5.4) provided that the repeatability criterion in point 8 is satisfied.

7. *Confirmation of the VC*

In cases where the VC released by materials and articles into the foodstuffs as calculated under point 6, exceeds the criterion in Article 2, paragraph 2 of the Council Directive 78/142/EEC of 30 January 1978, the values obtained in each of the two determinations (5.4) must be confirmed in one of three ways:

(i) by using at least one other column (4.3) having a stationary phase of different polarity. This procedure should continue until a chromatogram is obtained with no evidence of overlap of the VC and/or internal standard peaks with constituents of the sample of foodstuff;

(ii) by using other detectors, e.g. the micro-electrolytic conductivity detector*;

*See *Journal of Chromatographic Science* (volume 12), March 1974, page 152.

(iii) by using mass spectrometry; in this case, if molecular ions with parent masses (m/e) of 62 and 64 are found in the ratio of 3 : 1 it may be regarded with high probability as confirming the presence of VC. In case of doubt the total mass spectrum must be checked.

8. *Repeatability*

The difference between the results of two determinations (5.4) carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, must not exceed 0·003 mg VC/kg of foodstuff.

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REVIEWS OF RECENT PUBLICATIONS

Food Safety. Edited by H. R. Roberts. Wiley-Interscience, New York. 1981. pp. xiii + 339. £25.05.

This book deals with the basic question of the safety of the food supply. While absolute food safety is an impossibility, since virtually all food components represent some risk to some part of the population, the Editor, in his introductory chapter, attempts to put the problem in perspective. Dr Roberts, who was Director of the FDA Bureau of Foods before becoming Vice-President, Science and Technology, for the National Soft Drink Association, ranks five classes of food hazards from greatest to least risk as: (a) foodborne hazards of microbial origin, (b) nutritional hazards, (c) environmental contaminant hazards, (d) food hazards of natural origin and (e) food and colour additive hazards. He points out that the public conception of the relative risks is generally in the reverse order, and he puts much of the blame for this on the tendency of some scientists to seek popular attention, through the media, for unsubstantiated conclusions, prior to peer review and confirmatory research. He may well be missing the point here: many consumers accept that the regulators recognize hazards a–d and yet mistrust official attitudes on food and colour additives. They are concerned that studies on these additives often fail to yield unequivocal results but forget that consumer expectation and demands in relation to food appeal are a major reason for the use of colourings and many other food additives.

This contribution, which also comments briefly on the history of food safety, especially in the USA, introduces a systematic consideration of the individual hazards in relation to the overall picture of food-supply hazards. Subsequent chapters written by various toxicologists deal separately with the five classes of food hazards defined above. Each chapter discusses in some depth the sources and types of hazard, gives examples, outlines US regulatory controls where applicable and suggests methods of minimizing the problems. The chapter on 'Nutritonal hazards', written by V. J. Stults, provides an excellent introduction to the concept of food-component interaction.

A final chapter, by J. Doull, is entitled 'Food safety and toxicology'. This discusses the increasing strain on the science of toxicology resulting not only from demands on manpower and facilities but also from external and internal criticism of the credibility of animal-to-human extrapolation and retrospective epidemiology studies. There are brief treatises on science and public policy, the distinction between toxicity testing and safety evaluation, risk assessment techniques and the Delaney Clause. The author sums up by advocating a national food safety policy setting priorities based on risks rather than on politics and emotion. The importance of flexibility in regulatory policy, peer review, public participation and 'consumer education' are all stressed.

The tables and diagrams that supplement this text are well presented and the chapters are backed by useful lists of references. A sense of proportion and a sense of humour are maintained throughout—this is not a book by, or for, fanatics. The only background required is an interest in food safety and the text makes pleasant, well-balanced and informative reading.

Saccharin. A Report. By M. F. Cranmer. Edited by G. H. Scherr. Pathotox Publishers, Inc., 1980. pp. xiii + 586. $40.12.

Readers unversed in the saga of saccharin will find this book perplexing. In the absence of editorial comments or publishers' notes, one has to search for a sentence on page 8 and a paragraph on page 244 before deducing that the publication is in fact a report, written in 1978, by a former director of the National Center for Toxicological Research (NCTR), on the saccharin 'controversy' for the Office of Science in the Food and Drug Administration. The author was requested to undertake seven tasks or 'charges', including a review of experimentally induced bladder cancer in animals, an evaluation of work then in progress at NCTR and elsewhere, identification of further relevant research on saccharin and other chemicals likely to cause experimental bladder carcinogenicity and finally an evaluation of existing information and recommendations, if any, for further studies on the mutagenicity of saccharin.

The first part of the report consists mainly of a detailed analysis of the 18 or so long-term saccharin studies already completed, or nearing completion, by 1978. The comments of the National Academy of Science committee (1974) and other interested parties, especially the Calorie Control Council, as well as additional comments by the author are listed after each study, highlighting the movement of saccharin from the scientific to the political arena. For the reader, this section provides a variety of information, from identifying political pressure groups to exposing the inadequacies of many toxicological studies.

The second half of the book is the author's response to the seven FDA charges. The unorthodox presentation, the rambling style and the overwhelming use of jargon renders the result at best confusing and at worst unintelligible. This is a pity, because most of the information on saccharin toxicology up to 1978 is to be found somewhere in this labyrinth. Some of the sections are misguided. For example, was work in progress at other institutions (charge 2) best reviewed by submitting a verbatim transcript, warts and all, of a 1977 Toxicology Forum meeting on saccharin? And why did the author devote only two pages to the research proposed by his former institute (charge 3) when over 100 pages are given over to his own proposals for further work? Exactly what these are, however, is almost impossible to extract from the

saga of posed questions, repetitive background and asides on toxicological philosophy. After a tedious search the following proposals were identified: the effects of saccharin exposure *in utero* on bladder permeability, morphology and hyperplasia and the effects of saccharin on urine composition, magnesium ion flux, epithelial DNA synthesis and DNA repair after carcinogen treatment. Comparisons of Na saccharin with acid saccharin, 4-ethylsulphonylnaphthalene-1-sulphonamide and xylitol were suggested, with emphasis on the physiological effects these compounds can have in rats and in mice. In relation to mutagenicity, the author concluded that saccharin was unlikely to be a carcinogen, but recommended that work on the mutagenicity of urine from saccharin-treated animals should be pursued, and suggested further work on saccharin-induced enhancement of mutagenicity by bladder carcinogens. Finally, epidemiology data were reviewed, including a critique of the 1977 Canadian study, which purported to show an association between artificial sweetener consumption and bladder cancer.

Specific scientific criticisms of the report include the imprecise identification of scientific objectives and some incorrect references throughout the book. More immediate are the innumerable typographical errors, sloppy proof-reading, absent index and the overwhelming confusion of the text.

It is to be hoped that the FDA found less difficulty in digesting this treatise than did the reviewer. The book was apparently for 'everyone who uses an artificial sweetener'. They will need to be dedicated to the quest for information.

Mycotoxins and *N*-Nitroso Compounds: Environmental Risks. Edited by R. C. Shank. CRC Press, Inc., Boca Raton, FL. Vol. I, pp. 285; Vol. II, pp. 235; $74.95 each in the USA ($84.95 elsewhere).

The presence of mycotoxins and *N*-nitroso compounds (particularly nitrosamines) in food and feed are now widely accepted as major risks to human and animal populations. The editor has attempted to cover both classes of compounds together in two volumes where one volume for each class might have made for clearer and more logical presentation.

This is especially true for the sections dealing with mycotoxins. It is encouraging to find books devoted to the occurrence and properties of mycotoxins that are not concerned solely, or at least in the major part, with aflatoxin but these volumes do not greatly extend previously published knowledge. Details of the occurrence and analysis of mycotoxins and toxicity to humans and animals are presented in volume I. Volume II presents chapters dealing in the main with the metabolism, pharmacokinetics, biochemical effects and mode of action of individual groups of mycotoxins, but there is little or no cross-referencing to other chapters in the book. This fragmentary approach does little to help the reader to appreciate the relevance of mycotoxins as an environmental risk. However, the information in the individual chapters is well presented, very detailed and extensively referenced. The opening chapter of volume I includes a table in which are listed compounds that are probably involved in mycotoxicoses. It is a pity that two important groups mentioned, the penitrems and sporidesmins are barely mentioned in later chapters.

Regarding the coverage of *N*-nitroso compounds, four chapters (all in volume I) deal with the occurrence, toxicity and carcinogenicity, human responses and assessment of carcinogenic risk of *N*-nitrosamines in admirable if somewhat dated detail. These chapters are well organized and lucid and display some excellent features of which the most notable is the brevity and openness of the section on mechanisms of action. Here for once are authors (Dr Shank himself in conjunction with Professor Magee) who have resisted the temptation to reiterate the time-honoured and unsubstantiated speculation so often repeated in books of this kind. The paucity of information on human responses to *N*-nitroso compounds is also accurately reflected by the mere five pages devoted to this chapter.

***N*-Nitroso Compounds: Analysis, Formation and Occurrence.** Edited by E. A. Walker, L. Griciute, M. Castegnaro & M. Börzsönyi. IARC Scient. Publ. no. 31. International Agency for Research on Cancer, Lyon, 1980. pp. xxvi + 841. Sw. fr. 70.00 (available in the UK through HMSO).

This publication presents the proceedings of the VIth International Symposium on *N*-nitroso compounds held in Budapest in October 1979 and contains a large number of short papers on a wide variety of subjects. Separate sections are devoted to nitrosamine chemistry and formation, analysis, occurrence and experimental pathology. A short list of subcommittee recommendations for future work in each of these subject groupings completes the volume.

Publications of this type draw together a diversity of research approaches to what is usually a loosely defined subject area and the resulting breadth of scope has much to offer to those actively involved in any area considered. Such is the case with this book. Striking work on nitrosamine chemistry—including the first successful synthesis of an α-hydroxynitrosamine—stimulates the reader's imagination. Elegant new bioassay systems are described and only the analysis area seems stagnant. Although disturbing evidence is offered of the ever-widening range of situations in which nitrosamines seem to occur, the all-important methods for detecting non-volatile nitrosamines in the human environment and diet remain elusive. Altogether this conference clearly served the basic function of such gatherings—to inform and to stimulate.

Published proceedings of such meetings tend to have certain weaknesses, however, and this book is no exception. Very little can be gained unless one is prepared to study each paper in some detail. There is an absence of cohesion in the subject matter and the ease with which any information can be extracted is very dependent on the style of the individual authors. Some lecturers at these gatherings are anxious to give away as little as possible to their competitors, and this conference clearly had a few of those. Some authors want to give as many lectures as possible and so dilute one good piece of work into several trivial bits; again this conference had some of those. Some

authors have nothing original or worthwhile to say and there are even some of those.

But when all is said and done these meetings are essential and valuable. Perhaps one day a group of editors will assemble who have the courage to present the reader with refined gold rather than crude ore.

Handbook of Toxic and Hazardous Chemicals. By M. Sitting. Noyes Publications, Park Ridge, NJ, 1981. pp. xxi + 729. $64.00.

Increasing concern over the toxic hazards presented by chemicals at all stages from manufacture to ultimate disposal has generated a need for books that attempt to summarize all salient information under one cover. The above volume presents data on nearly 600 chemicals, including all those for which TLVs are allocated or proposed by ACGIH, that have been considered in the NIOSH Standards Completion Program or that are EPA priority toxic water pollutants. Most chemicals classified by the EPA as hazardous wastes or hazardous substances, or reviewed in EPA Chemical Hazard Information Profiles or NIOSH Information Profiles, are also covered. It is aimed at chemical manufacturers, safety equipment producers, toxicologists, industrial safety engineers, waste disposal operators, health care professionals and others who may have to make responsible decisions about chemical exposure.

For each chemical the data includes a chemical description, CAS, NIOSH Registry and UN code numbers, US Department of Transportation hazard classification, synonyms, potential exposure, incompatibility with other materials, US permissible exposure limits and determination in air and in water, routes of exposure, harmful effects and symptoms, points of attack (i.e. organs or systems affected), medical surveillance, first aid, personal protective methods, respirator selection, suggested disposal method, and (sometimes) a few important references. Those that have been designated carcinogens, hazardous substances, hazardous wastes or priority toxic pollutants by the various US federal agencies are also noted as such.

Although obviously intended primarily for American readers, much of the information presented is of worldwide application. It is commendably up to date by the standards of most text books, citing many 1980 references. For each chemical covered the information is more comprehensive than that in other works of reference commonly used for the same purpose. However, space considerations have often led to unduly brief descriptions of toxicological effects. Emphasis appears to have been given to acute effects and symptoms that have been observed in man, and effects so far reported only from chronic exposure of animals (apart from carcinogenicity) are often omitted. Thus, the entry for di-(2-ethylhexyl) phthalate records only that it can cause eye and mucosal irritation, nausea and diarrhoea, and omits any mention of the hepatic, testicular and teratogenic effects demonstrated in animals. Possible teratogenicity is also omitted from the entries for formamide and dimethylformamide, while that for di-*tert*-butyl-*p*-cresol (BHT) records only an oral LD_{50} in rats and a lack of toxicity to goldfish! There are errors of commission as well as omission—for example, a statement that no tumours were found in dogs given 4,4′-methylene-bis(2-chloroaniline) (MOCA).

Possibly if space-saving was the principal aim, details such as potential exposure (which includes the main uses of each compound) could have been omitted. As long as it is not relied on as the sole source of toxicological information, however, the book will probably be of value to a wide audience in the chemical and other industries for its succinct presentation of data, much of which has not before been collected in one manageable volume.

Carcinogenesis—A Comprehensive Survey. Vol. 6. **The Nitroquinolines.** Edited by T. Sugimura. Raven Press, New York, 1981. pp. viii + 159. $34.00.

The importance of the nitroquinolines in a variety of industrial processes and the value of 4-nitroquinoline 1-oxide as a model for the study of carcinogenic mechanisms have together resulted in a considerable accumulation of data on this group of compounds. This small volume attempts to present a comprehensive review of this experimental material.

The six chapters deal in turn with the chemistry and the metabolism of 4-nitroquinoline 1-oxide and related compounds, with mutagenicity in prokaryotes and genotoxicity in eukaryotes, and with cell transformation *in vitro* and carcinogenesis studies in experimental animals. Each reviews the published work in detail and provides an impressive list of references, including some from 1980.

Unfortunately, however, this is not a particularly readable book. The fact that neither Editor nor contributors are basically English speaking seems sometimes to have left its mark, but the problem is less one of style than of a multiplicity of abbreviations. It is hard to see how the fairly wide use of abbreviations could have been avoided, and for the most part the standard forms used will be familiar to readers already well versed in this field, but for others a separate glossary would have been more helpful than the definitions provided in the texts of individual chapters.

Health Impacts of Polynuclear Aromatic Hydrocarbons. Environmental Health Review No. 5. Edited by A. W. Pucknat. Noyes Data Corporation, Park Ridge, NJ, 1981. pp. x + 271. $39.00.

Analytical Chemistry of Polycyclic Aromatic Compounds. By M. L. Lee, M. V. Novotny and K. D. Bartle. Academic Press, London, 1981. pp. xi + 462. £33.60.

These two books identify and examine in detail one area of current concern for the environment and our health—the polynuclear aromatic hydrocarbons (PAHs)—a group of seemingly ubiquitous environmental contaminants with established carcinogenic properties.

Health Impacts of Polynuclear Aromatic Hydrocarbons reviews the evidence collected largely by United States Government Agencies in three main

areas: the fossil fuel sources of PAHs, their fate in the environment and their effects on human health. There are also chapters on the physical and chemical properties of the more common PAHs, analytical methods, and a description of some health protection criteria adopted in the USA. Despite the claims of the publishers to provide up-to-date information, there are very few references beyond 1978 and the extensive contents list does little to compensate for the lack of a subject index. Although most of the environmental data given relate to the USA, the book is a useful monograph for all students of the environment and should provide valuable background information for toxicologists involved in PAH research.

Analytical Chemistry of Polycyclic Aromatic Compounds describes analytical procedures that have been developed for complex polynuclear aromatic mixtures which contaminate, or originate in, air, water, sediments, food, tobacco smoke and fossil fuels. Three introductory chapters outline the physical and chemical properties, occurrence, and the toxicology of polycyclic aromatic compounds. Critical reviews of specific aspects of analysis for polycyclic aromatic compounds follow. The topics covered are collection, extraction, and fractionation, column, paper and thin-layer chromatography, high-performance liquid chromatography, gas chromatography, mass spectrometry, ultraviolet and luminescence spectroscopy, nuclear magnetic resonance spectroscopy, and fourier-transform infra-red spectroscopy. A final chapter highlights the analysis of methylchrysenes by some of the methods discussed in the preceding chapters, and there are several useful appendices giving data on the structures and carcinogenicity of PAHs and heteroaromatic compounds. Detailed theoretical accounts are kept to a minimum throughout the book, and the emphasis is on practical working problems. Relatively up-to-date references, clear print and a subject index all help to make this a valuable account of polynuclear hydrocarbon analysis which should be of use both to workers who are new to polynuclear aromatic compound analysis and to those who are familar with this area, but in need of a compendium of current methods.

Progress in Drug Metabolism. Vol. 5. Edited by J. W. Bridges & L. F. Chasseaud. John Wiley & Sons Ltd, Chichester, 1980. pp. ix + 358. £28.50.

This book is the fifth in a series which aims by critical reviews to keep the reader up to date with developments in various aspects of xenobiotic metabolism (*Cited in F.C.T.* 1981, **19**, 775). The present volume consists of five reviews each with a list of references, and a subject index for the entire volume.

The first chapter by J. C. Connelly and J. W. Bridges deals with the distribution and role of cytochrome *P*-450 in mammalian extrahepatic organs. Cytochrome *P*-450 is of course the generic term for a family of haemoproteins that act as the terminal oxygenases in the metabolism of both endogenous (e.g. steroids, vitamins, fatty acids) and exogenous (xenobiotic) substances. Although cytochrome *P*-450 levels are generally much lower in extrahepatic tissues than in the liver the importance of these sites of metabolism should not be overlooked. The second review by C. H. Walker deals with species variations in hepatic xenobiotic metabolizing enzymes with particular reference to cytochrome *P*-450-dependent mixed-function oxidases, epoxide hydratase and UDP-glucuronyltransferase. Enzyme activities determined with subcellular fractions of liver homogenates are catalogued, and the sources and scale of variation, *in vivo* studies and the use of microsomal systems as models for whole animal metabolism are considered.

The third chapter by H. B. Hucker, K. C. Kwan and D. E. Duggan deals with the pharmacokinetics and metabolism of non-steroid anti-inflammatory drugs. A wide range of compounds are considered including salicylates (including aspirin), arylacetic acids, 2-arylpropionic acids, *N*-heterocycles (including phenylbutazone) and other heterocycles and the review is well illustrated with chemical structures and biotransformation pathways. The next chapter by V. Marks and coworkers considers the monitoring of drug disposition by immunoassay. Radioimmunoassays, enzyme immunoassays and different types of fluorescence immunoassays are among the methods described together with examples of their application.

The final chapter by P. G. Watanabe, J. C. Ramsey and P. J. Gehring is entitled "Pharmacokinetics and metabolism of industrial chemicals" and is highly recommended to anyone interested in the toxicological evaluation and risk assessment of industrial chemicals. The authors briefly review basic pharmacokinetic principles and then consider kinetic models of the formation of reactive metabolites and of their interactions with cellular macromolecules such as DNA, RNA and protein that may ultimately initiate carcinogenesis. The industrial chemicals considered include styrene, 2,4,5-trichlorophenoxyacetic acid, 1,4-dioxane, vinylidene chloride and tetrachloroethylene.

As with previous 'Progress' volumes this book is to be recommended both for general reading and for any of the five specific areas covered. Oh, but the price!

Information Resources in Toxicology. By P. Wexler. Elsevier/North-Holland Inc., New York, 1982. pp. xv + 333. Dfl. 105.00.

Information Resources in Toxicology is a very timely review of a complex and rapidly developing area. Mr Wexler has presented a comprehensive coverage of a multi-faceted subject so that the practising toxicologist, the information specialist and the layman can locate and evaluate relevant information sources. It is not intended to structure actual searching procedures for specific toxicological data but rather to broaden the scope of the search to a wider variety of potential resource material.

Seven major categories of information resources and processing are selected by the author; these are reference sources, organizations, legislation and regulations, international activities, education, information handling and journal articles. Within each of these categories are separate chapters containing brief descriptions of sources of general or specific information. Approximately half of the book (over 1200 entries) is devoted to listing books, monographs,

periodicals and journal articles that provide toxicological information. Many of these sources are classified into groups covering carcinogenicity, mutagenicity, teratogenicity, biotoxins, clinical toxicology, drugs and cosmetics. Other types of information sources listed are abstracts journals, indexes, current awareness publications, directories and (almost exclusively US) computer data bases. There are also lists of US governmental and nongovernmental organizations concerned with toxicology. Descriptions are also included of relevant US regulations. Another section lists the universities in the United States that offer graduate courses in toxicology. In an appendix US Poison Control Centers are tabulated by State. Finally, two subject indexes are provided, one to cover the section on journal articles and the other dealing with other listed sources of data.

Obviously the book is directed towards the American market, and many of its sections are therefore of limited use to those working elsewhere. However, in summary, Phillip Wexler has dealt with an extremely diverse and dynamic subject and presented lucid descriptions of resources that will assist in the search for toxicological information. It is to be hoped that this publication will be updated regularly in order to maintain its effectiveness.

An Atlas of Laboratory Animal Haematology. By J. H. Sanderson and C. E. Phillips. Oxford University Press, Oxford. 1981. pp. 473. £75.00.

Many of the problems facing laboratory animal haematologists stem from difficulties in the accurate recognition of cell types in both the circulating blood and bone marrow of the various species they are called upon to examine. The uncertainties that arise may lead to errors and make comparisons between results from different laboratories difficult. These problems have been recognized for some time and the authors of this book deserve credit for their attempts to provide a much needed source of reference.

The range of species covered is not exhaustive but includes many of those that are commonly, and less commonly, used including rats, mice, dogs, guinea-pigs, hamsters, rabbits, marmosets, sheep, cattle and pigs. To provide a reference point for those more familiar with human haematology, man is also included. The animal material was obtained from animals housed at the ICI site, Alderley Park, Cheshire and is from normal animals, the use of pathological material being deliberately omitted.

Each chapter deals with a single species and begins with a summary of general haematology which includes a brief description of the major features of each cell type and the common haematological values of circulating blood. Romanowsky-stained blood and marrow films are photographed at magnifications of × 480 and × 750 (sometimes at × 300) and the overall quality is high, with an accurate colour rendition. There is a certain amount of repetition of examples showing similar features and one feels that the number of photographs could have been reduced without affecting the usefulness of the book. It was surprising to find in each chapter a number of photographs of blood films taken through Nomarski contrast optics; these added little to an understanding of the cells illustrated and must be of very limited practical value considering the rarity of this equipment in routine haematology laboratories.

The photographs are presented on one page with an accompanying brief text pointing out the salient features of each. On the facing page are line drawings of the photographs with the cells of interest outlined and labelled. This often comprises only one or two, and on occasions no, labelled cells in the line drawing. Unfortunately this approach does not always work well, and in many cases matching up the line drawing with the photograph proved to be an error-prone exercise, particularly in the case of the very cellular marrow films, unless one could recognize *a priori* the cell in question.

The atlas ends with a very short section on methods of preparation of blood and marrow films and some techniques of examination. Finally there is a short glossary, and a bibliography of 11 references.

A major criticism of this book is the amount of wasted space. By omitting the Nomarski contrast photographs which are of little practical value, reducing the number of photographs showing similar features, and using a different method of identifying cells, its volume could have been reduced by more than half. Within the limited terms of reference set by the authors, the atlas is reasonably successful in its aim of presenting "an easily accessible source of information to anyone who is called upon to study the haematology of animals in a laboratory environment". However, most of those working on animal haematology are concerned with the detection and interpretation of abnormalities, and therefore a book that deals solely with normal tissues will inevitably have rather limited appeal. Despite these shortcomings, and assuming the price does not prove prohibitive, the atlas will be useful to clinical haematologists encountering animal haematology for the first time, and to those already working in the field who wish to examine species that are unfamiliar to them.

Stereological Methods. Vol. 1. **Practical Methods for Biological Morphometry.** By E. R. Weibel. Academic Press Inc. (London) Ltd, London, 1979. pp. xvi + 415. £27.60.

Stereological Methods. Vol. 2. **Theoretical Foundations.** By E. R. Weibel. Academic Press Inc. (London) Ltd. 1980. pp. xiv + 340. £26.00.

In recent years there has been a growing trend in all fields of the biological sciences to present quantitative rather than qualitative results. This is particularly evident in areas that use microscopy as an analytical tool, where a scoring system has often been adopted to express the extent or frequency of observed changes. One of the reasons for the use of this semiquantitative approach rather than precise quantitation stems from the often complex relationship between measurements obtained from a two-dimensional section seen through the microscope and those of the original three-dimensional structure. It is at this

problem that stereological methods are specifically aimed.

The discipline of stereology, a term coined as recently as 1961, is a branch of applied mathematics and therefore may not be readily understandable to those who stand to benefit most from its application (pathologists and electron microscopists for example). To overcome this, the author named above has split his work into two volumes.

Volume 1 is concerned mainly with the practical aspects of stereology, although it also includes an adequate but nevertheless elementary coverage of the theoretical principles upon which the methods are based. The second volume covers similar ground but from a much more theoretical standpoint. This is a sensible approach, which on the whole works well, allowing those who are interested mainly in grasping the essentials of stereology and how it is applied to do so without being distracted by theoretical considerations. Volume 2 is there for consultation if more detail is required, and will be of particular use to those designing schemes to suit individual needs.

Both volumes begin with an introductory chapter on the concepts and history of stereology. Subsequent chapters in volume 1 cover stereological principles, sampling, point counting and the problems of particle size and shape. The final chapters give guidance on the planning of stereological studies, and describe model cases demonstrating the variety of problems that may be tackled with these techniques. A good example of the type of problem amenable to stereological analysis is given by the first model, for determining the volume, surface area and numbers of various subcellular components of liver cells. Other examples include the analysis of different types of muscle cells, the correlation of the thickness of the lung-tissue barrier with oxygen diffusion and of alveolar curvature with lung inflation, and studies of plasma cell differentiation, the structure of cancellous bone, and the secretory state of the adrenal cortex. Volume 2 treats in more depth many of the principles covered briefly in volume 1 to allow those with an aptitude for mathematics to understand the often complex derivations of the relatively simple stereological formulae required for the design of new schemes.

Both volumes contain useful appendices defining the symbols and terms used and providing good up-to-date bibliographies. Together, the pair provide what is probably the best single source of reference currently available on the biological applications of stereology and must be strongly recommended to all those engaged in microscopy who wish to become familiar with this most useful analytical method.

BOOKS RECEIVED FOR REVIEW

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Environmental Health Chemistry: The Chemistry of Environmental Agents as Potential Human Hazards. Edited by J. D. McKinney. Ann Arbor Science Publishers, Ann Arbor, MI/The Butterworth Group, Sevenoaks, Kent, 1981. pp. xiv + 656. £27.40.

Chemical Mutagenesis, Human Population Monitoring and Genetic Risk Assessment. Edited by K. C. Bora, G. R. Douglas & E. R. Nestmann. Elsevier Biomedical Press, Amsterdam, 1982. pp. xxiv + 364. Dfl. 190.00.

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Genetic Engineering of Microorganisms for Chemicals. By A. Hollaender, R. D. DeMoss, S. Kaplan, J. Konisky, D. Savage & R. S. Wolfe. Plenum Publishing Corporation, New York, 1982. pp. xiii + 485. $55.00 (+20% outside USA).

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 81. Edited by F. A. Gunther & J. D. Gunther. Springer-Verlag, New York, 1981. pp. viii + 172. $22.80.

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Fd Chem. Toxic. Vol. 20. pp. 617 to 621, 1982
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Pergamon Press Ltd

Information Section

ARTICLE OF GENERAL INTEREST

ARE WE AT RISK FROM LEAD?—PART 1

The use of alkyllead as an anti-knock additive in petrol has been the subject of much controversy particularly in recent months. Lead-based petrol additives are just one of a wide range of uses of this metal which have resulted in its wide dispersal in the environment and while the effects of acute exposure to lead are well established, effects at low levels are less certain. In this and a subsequent review we intend to examine whether lead levels to which the general population is exposed today are likely to be associated with adverse effects.

Blood levels in the general population

An EEC survey of nearly 18,000 subjects recently revealed a median blood-lead (PbB) level of 13 μg/dl*, although slightly more than 2% exceeded 30 μg/dl and 1·05% (184 subjects) exceeded 35 μg/dl (*Human Biological Monitoring of Industrial Chemicals 4. Inorganic Lead*, by L. Alessio & V. Foa; EUR 6982 EN, Luxembourg, 1980). These values were within the limits specified as not requiring notification to the EEC Commission, i.e. levels not exceeding 20, 30 and 35 μg/dl for 50, 90 and 98% respectively of the people examined (*Off. J. Europ. Commun.* 1977, **20,** (L105) 10). UK results, published in the 'Lawther report' (*Lead and Health*, HMSO, London, 1980), showed that the proportion of adults in major urban areas with levels exceeding 20 μg/dl ranged from 32% in Glasgow down to 3–6% in four areas of London. Children of lead workers and adults and children living near main roads tended to have higher levels, and 35% of children and 16% of adults living near the M4 at Hillingdon were above the 20 μg/dl level, as were 31% of children living near a main road in Tower Hamlets.

Effects of low levels of lead on the haemopoietic system

At very low PbB levels, lead inhibits the action of the enzyme δ-aminolaevulinic acid dehydratase (ALAD), which converts δ-aminolaevulinic acid to porphobilinogen during the synthesis of haem (a constitutent of haemoglobin and of the cytochromes). It is doubtful whether this inhibition has any threshold, but if so it is well below 10 μg/dl. In nine children with PbB levels below 14 μg/dl, ALAD activity was inhibited by more than 40% in only one, whereas at PbB levels of 15–24 μg/dl 73% of 37 children showed more than 40% inhibition and 8% showed more than 70% inhibition (Zielhuis, *Int. Archs occup. Hlth* 1975, **35,** 1). However, as excretion of ALA in the urine is not significantly increased at PbB levels below about 40 μg/dl, it is thought that the enzyme has a large reserve capacity, and its inhibition is of physiological significance only at levels sufficient to increase the build-up of ALA (*Federal Register* 1977, **42,** 63076).

A more serious effect of low PbB levels is the accumulation of erythrocyte protoporphyrin (EP). This occurs above threshold PbB levels of about 15–20 μg/dl in children and women, and 30–40 μg/dl in men (*Federal Register* 1977, **42,** 63076; *ibid* 1978, **43,** 54354). This is the result of decreased efficiency of iron insertion into protoporphyrin, the final stage of haem synthesis which takes place inside the mitochondria. Accumulation of protoporphyrin in neural tissue (chick dorsal root ganglion cells) has also been demonstrated *in vitro*, and formation of cytochrome *P*-450 in the liver may be inhibited by the same mechanism. Because EP elevation indicates an abnormal impairment of various cell functions, which may have particular implications for the functioning of neural and hepatic tissues, the EPA has used the lowest determined threshold (15 μg/dl) for this effect as the basis for the recent US air quality standard for lead (*Federal Register* 1977, **42,** 63076; *ibid* 1978, **43,** 46246). EP levels are generally regarded as elevated when they exceed 60 μg/dl (Posner *et al.* in *The Biogeochemistry of Lead in the Environment*, Part B, p. 204. Edited by J. O. Nriagu, Elsevier/North-Holland Biomedical Press, Amsterdam, 1978).

More recently it has been reported (Cavalleri *et al. Envir. Res.* 1981, **25,** 415) that children aged 3–11, including 165 living near a lead smelter and 89 from a village 4 km away, showed a graded increase in free erythrocyte porphyrin (FEP) at PbB levels in the range of only 10–25 μg/dl. [More than 90% of FEP consists of protoporphyrin IX (Baloh, *Archs envir. Hlth* 1974, **28,** 198).] Iron deficiency may also cause elevated FEP levels, but in this study there was no difference between the two groups in haematocrit, haemoglobin or serum iron concentrations. All of the village children had PbB levels below 20 μg/dl, whereas of those near the smelter 23% were in the range 21–30 μg/dl and 3% were in the range 31–40 μg/dl. Only 4% of the village children had an FEP level exceeding 60 μg/dl, compared with 44% of those near the smelter. The mean + 2SD value (62 μg/dl) calculated from levels found in the village children was chosen as the FEP level above which values were considered to be elevated. This level was not exceeded by any of the 11 subjects from either location with a PbB level below 10 μg/dl. However, among children with PbB levels in the range 10–14, 15–19, 20–24 and ⩾ 25 μg/dl the percentage with FEP levels exceeding this limit increased progressively from 35 to 40, 48 and 67%, respectively. The authors of this study conclude that the no-response PbB level

*dl = 100 ml.

for FEP elevation in children seems to be below 10 μg/dl, a value only two-thirds of that used as a basis for the EPA standard.

At somewhat higher blood concentrations, lead begins to inhibit erythrocyte membrane Na^{+}-K^{+}-ATPase and pyrimidine 5′-nucleotidase. In children these effects become significant at 20–30 μg Pb/dl (Angle & McIntire, *Toxic. appl. Pharmac.* 1979, **48**, A116). The former may lead to a shortened erythrocyte lifespan. Anaemia, i.e. a decrease in haemoglobin and erythrocyte numbers may become significant in iron-deficient children at about 40–50 μg Pb/dl, although in normal adults the threshold is probably about 70–80 μg Pb/dl (*Environmental Health Criteria 3: Lead*, WHO, Geneva, 1977; Zielhuis, *loc. cit.*). Coproporphyrin excretion in the urine also increases at about 40 μg/dl (*Federal Register* 1977, **42**, 63076).

Effects on the nervous system

Children. At higher blood levels than are required to affect haem synthesis, lead can cause peripheral neuropathy and encephalopathy. What is more controversial is whether low PbB levels, in the range more commonly encountered in the general population, may affect children's intelligence, behaviour, attainment and performance. We have reviewed some of the earlier evidence on this subject (*Cited in F.C.T.* 1975, **13**, 277; *ibid* 1975, **13**, 588) and more recent reviews are also available (Bornschein *et al. CRC Crit. Rev. Toxicol.* 1980, **8**, 43; Repko & Corum, *ibid* 1979, **6**, 135; Rutter, *Devl Med. Child Neurol.* 1980, **22**, Suppl. no. 42, p. 1). The latter author concludes that "although the findings are somewhat contradictory, the evidence suggests that persistently raised PbB levels in the range above 40 μg/100 ml may cause slight cognitive impairment (a reduction of one to five points on average) and less certainly may increase the risk of behavioural difficulties. There are pointers that there may also be psychological risks with lead levels below 40 μg/100 ml, but the evidence on this point is inconclusive so far". Professor Rutter was also a member of the Lawther Committee, the DHSS Working Party on Lead in the Environment (*Lead and Health*, HMSO, London, 1980), which in the same year concluded that "there is no convincing evidence of deleterious effects at blood lead concentrations below about 35 μg/dl".

One complication in studies on children is that the subjects often have high PbB levels through pica, a habit that is more frequent in the less intelligent and behaviourally disturbed. It cannot therefore automatically be concluded that their lower intelligence is a result of their lead exposure (Rutter, *loc. cit.*). Families that are disadvantaged by low intelligence may also be exposed to lead from other sources, such as peeling paint or leaded water pipes (commoner in older houses) and may be more likely to live by main roads or in industrially polluted areas. Therefore socio-economic factors must be carefully controlled to prove that there is a causal relationship between lead exposure and lower intelligence.

The most controversial study in this regard (Needleman *et al. New Engl. J. Med.* 1979, **300**, 689) compared 58 children (about 7–8 yr old) with tooth (dentine) lead levels above 20 ppm with 100 others in whom mean tooth lead was below 10 ppm. Mean PbB levels (assessed in a limited number of the subjects some years before the study) were 35·5 ± 10·1 μg/dl and 23·8 ± 6·0 μg/dl for the two dentine-lead groups, respectively. The high dentine-lead group scored significantly less well on the Wechsler intelligence scale for children, particularly in the verbal tests; their mean verbal IQ was 99·3 and their performance IQ was 104·9, in comparison with 103·9 and 108·7 respectively in the low-lead group. The performance of the high lead children was also worse in three other measures of auditory or speech processing and a measure of attention. Analysis of covariance showed that none of these differences could be explained by any of the 39 other variables studied, including parental education, IQ and occupation. When an 11-point teacher's questionnaire was used to evaluate classroom behaviour, children with high dentine-lead levels were rated worse on all 11 scores, and significantly worse ($P < 0·05$) on nine of the scores. Furthermore, when the questionnaire was applied to 2146 of the 2335 children whose teeth were analysed, the frequency of non-adaptive classroom behaviour was shown to increase in a dose-related manner with dentine-lead level. The children in this case were divided into six subgroups, with dentine lead ranging from below 5·1 ppm in the first to above 27 ppm in the sixth. With increasing dentine lead an increasingly greater proportion of each subgroup was in general classified as distractable, not persistent, dependent, disorganized, hyperactive, impulsive, frustrated, day-dreamers, unable to follow simple directions or sequences, and below average in overall functioning.

One objection that was raised to the published report of this study was that pica was not used as a covariate, although it occurred at a much higher frequency in high- than low-lead children (29 *v.* 11%). However, Needleman (personal communication to Rutter, *loc. cit.*) later reported that the incidence of pica was associated with verbal IQ in the low but not in the high lead group, and with performance IQ in both groups, but at levels short of statistical significance. The difference in IQ could not therefore be explained on this basis. The incidence of pica showed no relationship with the teachers' ratings of the children's behaviour. Unfortunately the latter were controlled for socio-economic and other variables only in the case of the two original low (< 10 ppm) and high (> 20 ppm) dentine-lead groups and so for the intermediate groups in the larger study a causal relationship between lead and poor performance in the behavioural ratings is therefore only presumptive.

The findings in Needleman's study of Massachusetts children were lent some support by another in Germany, which revealed a difference of some 5–7 points in mean IQ level between 26 children with a dentine-lead level below 3 ppm, and 26 with a level exceeding 7 ppm (Hrdina & Winneke, 1978 conference paper cited by Rutter, *loc. cit.*). However, this difference in IQ fell short of statistical significance. The groups were matched for age (7–10 yr), sex and socio-economic status, but were not controlled for other background factors.

In an extension of Needleman's study, spontaneous electroencephalograms were recorded for 19 of the high-lead and 22 of the low-lead children. On the

basis of computer-assisted spectral analysis of the electroencephalograph (EEG) data, the high-lead group had significantly increased amounts of low-frequency delta (0·5–3·5 Hz) activity over central parietal and occipital cortices and significantly decreased amounts of alpha (8–12 Hz) activity over parietal and occipital regions. When used alone, EEG or psychological variables could be used to distinguish between the two groups at levels significantly above chance, and the predictive powers of EEG and psychological features combined were greatly superior to either alone (Burchfiel *et al.* in *Low Level Lead Exposure: the Clinical Implications of Current Research*, p. 75. Edited by H. L. Needleman, Raven Press, New York, 1980). The lower amounts of alpha activity in the high-lead children might signify that brain maturation was somewhat delayed by lead since such activity tends to increase with age (Matuosek & Petersen, *Electroenceph. clin. Neurophysiol.* 1973, **35**, 603 cited by Benignus *et al. ibid* 1981, **52**, 240).

Two other recent studies have also revealed a correlation between body burden of lead and the electrical activity of the brain in young children. Otto *et al.* (*ibid* 1981, **52**, 229) studied slow cortical potentials during sensory conditioning in 63 children aged 13–75 months, mostly from low-income black families, of mean IQ 88·0 ± 13·4 (1SD) and with PbB levels in the range 7–59 μg/dl. In one test the children were made to listen to a series of standard (1 kHz) tones, interrupted at intervals by a tone of different frequency (2 kHz) while watching a cartoon film. In a second test the cartoon was periodically blacked out for 1·5 sec after onset of the 2 kHz tone. Younger children who would not watch cartoons were shown puppets instead, and those under 48 months usually sat on the lap of an adult. Slow-wave voltage, presumed to be an index of sensory conditioning, showed a significant relationship with age, but not with PbB levels during the first test. However in the second test slow-wave voltage varied as a linear function of PbB level, although the slope changed systematically with age. For children under 5 yr old it tended to be positive at low PbB levels and negative at PbB levels above 30 μg/dl, while for those over 5 yr it tended to be negative at low PbB and less negative or positive above 30 μg/dl. These latter findings suggest a decrease in attentiveness with increased PbB levels, and may perhaps lend support to Needleman's findings of greater distractability in children with high dentine-lead levels. However, the study can be criticized on the grounds that the cartoon represented a complex stimulus and the parameters measured are thought to be poorly developed in children under 6 yr of age (Cohen *et al. ibid* 1967, **23**, 77). The younger children's response may have been influenced by the adults on whose laps they were sitting and/or by the different stimulus presented. PbB rather than dentine lead was measured and therefore indicated lead exposure in the immediate past rather than long-term body burden. In a further experiment by this group (Benignus *et al. loc. cit.*) the same children were shown cartoons and their EEG power spectra and various measures used to compare electrical activity in the two sides of the brain were investigated. At PbB levels of up to about 15 μg/dl, increased lead was associated with a strong increase in the relative amplitude of synchronized EEG between left and right parietal in all frequency bands. The increase then levelled off, a slight decrease occurred between about 30 and 40 μg/dl but there was a sharp increase above 40 μg/dl. The authors suggest that as PbB approached 15 μg/dl some compensatory mechanism may have come into action which managed to offset effects, until it was overcome at levels of 40 μg/dl or more. However, in view of the number of subjects and the stepwise data analysis, they consider that replication would be needed to confirm the apparent biphasic nature of the response. Increased synchrony of the EEG could indicate an increase in the amount of information being processed (Surwillo, *Cortex* 1971, **7**, 246), and an increase in relative amplitude perhaps suggests that greater effort is required to assimilate this information (Maxwell *et al. Psychol. Med.* 1974, **4**, 274) on the part of children with a high burden of lead.

When the quality of the home environment of 52 children from the same population used to select participants in the studies by Otto *et al.* (*loc. cit.*) and Benignus *et al.* (*loc. cit.*) was investigated, a correlation was found between their PbB levels and maternal care and intelligence (Milar *et al. Am. J. ment. Defic.* 1980, **84**, 339). For those less than 30 months old, PbB levels of 31 μg/dl or more were significantly associated with a lower degree of emotional and verbal responsivity of the mother (her tendency to praise the child's behaviour) and of maternal involvement (encouragement of developmental advance, and structuring of the child's play periods). Such mothers were also more likely to shout at or otherwise express overt annoyance towards the child, and less likely to provide appropriate toys, to read stories at least three times weekly or to share a meal with their children, although the correlations in these cases fell short of significance. The IQ (assessed in the Quick test, a brief test of verbal knowledge) of the mothers of younger children with high PbB levels was also significantly lower (mean 75·0 *v.* 85·1 in mothers of younger low-PbB children). The mothers of the older high-PbB children showed similar tendencies, but none of the differences were statistically significant. It was concluded that both the low intelligence of the mother and the poor care-giving environment increased the possibility of the children finding and ingesting lead. The authors considered it most likely that lower maternal intelligence, the poor care-giving environment and the neurotoxic effects of lead all combine to affect intellectual development adversely. Therefore it is unlikely that the detrimental effects of lead on young children can be adequately assessed without taking these other factors into account.

The tabular data presented by Otto *et al.* (*loc. cit.*) also indicate that children with PbB levels above 30 μg/dl had a mean IQ nine points lower than those with PbB levels below 30 μg/dl [83·0 ± 14·0 (1SD) *v.* 92·0 ± 11·6]. From the above it would appear that this may have been an inherited difference, compounded by lack of maternal care, and not entirely a result of lead toxicity. [It might also be questioned whether differences in brain electrical activity could be a product of inheritance and upbringing, rather than of lead levels, but this is not a topic discussed in any of these papers. Whether the reported changes in EEG and evoked response in children with high lead

levels influenced IQ, learning ability or behaviour has still to be confirmed.]

A study of 166 children aged 6–12 yr, living near a leadworks in outer London, has also suggested that low PbB levels may affect scholastic ability (Yule *et al. Devl Med. Child Neurol.* 1981, **23**, 567). Their PbB levels (assessed some 9–12 months before psychological testing) were in the range 7–33 μg/dl (mean 13·52 μg/dl) and for the purposes of the study they were divided into four subgroups with PbB in the range 7–10, 11–12, 13–16 and 17–32 μg/dl respectively. There were significant associations between PbB levels and attainment scores in tests for reading, spelling and intelligence, although not mathematics. Even after standardization for social class these differences remained largely intact; reading accuracy and comprehension and spelling showed a graded decline with increasing lead levels, while for verbal IQ, performance IQ and full-scale IQ there appeared to be a threshold at a PbB level of 13 μg/dl (full-scale IQ = 103·32 and 102·68 in the two lower PbB groups and 95·75 and 85·70 in the two higher PbB groups). Allowing for age, sex and social class also failed to remove the correlation with PbB levels but did indicate that only a small proportion of the variance in intelligence was explained by PbB levels. The authors considered that further work paying greater attention to social factors would be necessary to confirm their findings, and this they are now undertaking.

One variable not considered by Yule *et al.* (*loc. cit.*) was parents' IQ, an omission rectified in two studies of black children in New York City (Ernhart *et al. Pediatrics, Springfield* 1981, **67**, 911; Perino & Ernhart, *J. Learn. Disabil.* 1974, **7**, 26). When 80 of these children were studied at ages 3–5, those with PbB levels of 40–70 μg/dl scored significantly worse on the general cognitive index and the verbal and perceptual performance subscales in the McCarthy Scales of Children's Abilities than did those with PbB levels of 30 μg/dl or less, even after regression analysis to eliminate potential contributions from age, birthweight and the parents' (usually the mother's) intelligence. PbB levels were, however, negatively related to the level of the parents' education. Lead also appeared to interfere with the normal correlation of children's intelligence with that of their parents, in that for children with PbB levels over 40 μg/dl the correlation was only 0·1. This was significantly different from the 0·52 for the children with lower blood lead levels, the latter value being close to the normal correlation of 0·5 (Perino & Ernhart, *loc. cit.*).

When 63 of the same children were studied 5 yr later by Ernhart *et al.* (*loc. cit.*), their PbB levels had decreased and become significantly less variable. As before, the children were divided into two groups with 'low' (10–30 μg/dl) and 'moderate' (40–70 μg/dl) PbB levels, and subjected to tests of intelligence (McCarthy Scales), a reading test (which varied according to the school attended) and teacher ratings on five behavioural scales. There was a significant correlation between present PbB levels and scores for reading ability ($P < 0{\cdot}002$), and McCarthy scale intelligence ($P < 0{\cdot}041$) and reading ability were also correlated with pre-school PbB levels. On the McCarthy scales children with moderate PbB levels scored significantly worse on the general cognitive index and verbal, perceptual-performance, memory and motor subscales. However, when the 'caretaking' parents' IQ (assessed using the Quick test), the parents' education and the children's sex were incorporated into the regression analysis, only scores on the general cognitive index and verbal and motor subscales remained statistically significant, and these only at the 5% level. Reading ability was no longer significantly related to either PbB measure, and the pre-school PbB level showed no correlation with present performance. Another surprising finding was that children with moderate PbB levels were less likely to be rated by their teachers as easily frustrated, hyperactive, impulsive and distractable than were those with low PbB levels, in direct contrast with Needleman's (*loc. cit.*) results, although like the children in Needleman's study they were more likely to day-dream. (However, in the present study none of the ratings differed significantly between the two groups]. When the relationship between the parents' IQ and the school-aged children's intelligence was reassessed on the basis of pre-school PbB levels, the scores of children with moderate PbB levels on the general cognitive index and on the verbal subscale both showed a 0·33 correlation with parents' intelligence. This was not statistically different from the 0·48 and 0·47 correlations in the low PbB group, in conflict with results 5 yr earlier.

Ernhart *et al.* (*loc. cit.*) consider it possible that a model incorporating a more sensitive measure of parent's intelligence and measures of other factors affecting child development would have reduced the correlation with lead levels even further. They further suggest that as only this and Needleman's paper have taken parents' IQ into account, all previous studies suggesting a relationship between children's performance and body lead levels should be regarded as suspect. [They do not cite the work carried out by Milar *et al.* (*loc. cit.*) and Otto *et al.* (*loc. cit.*) on pre-school-age children.] However, as Needleman *et al.* (*ibid* 1981, **68**, 894) subsequently pointed out, in reaching this conclusion Ernhart *et al.* ignored their own earlier findings (Perino & Ernhart, *loc. cit.*). They also omitted to compare IQ correlations between parents and children classified on the basis of present PbB, despite their recalculation on the basis of pre-school findings.

Lead workers. Although the study by Ernhart *et al.* might suggest that even PbB levels as high as 70 μg/dl have little if any effect on mental function, this is not borne out by studies of adult lead workers. When 42 such workers with PbB levels in the range 12·6–88·2 μg/dl (median 46·2 μg/dl) were compared with 22 workers in an oil mill, the lead workers performed significantly worse in tests of intelligence (the complete Wechsler Adult Scale), psychomotor ability and memory. Long-term memory, verbal and visuo-spatial abstraction and psychomotor speed were particularly affected (Grandjean *et al. Scand. J. Work Envir. Hlth* 1978 **4**, 295). Age and time of exposure to lead were not found to be significant confounding factors in this study. A group of 90 secondary lead smelter workers also performed less well than groups of 25 steel workers, 99 paper-mill workers and 93 farmers in behavioural tests used to assess central nervous system dysfunction, including those to measure ability to discern spatial relations, to manipulate sym-

bols according to a code, and to perceive objects in masked conditions. The decreases in test scores were not attributable to differences in age or education, but were highly correlated with increases in erythrocyte zinc protoporphyrin. [When lead is absorbed the Zn chelate of protoporphyrin accumulates (Lamola & Yamane, *Science, N.Y.* 1974, **186**, 936) and this form can be extracted and measured instead of FEP.] The majority of the lead smelter workers (61%) had PbB levels between 40 and 59 μg/dl, with a range in the group as a whole of below 40 to above 80 μg/dl (Valciukas *et al. Int. Archs occup. envir. Hlth* 1978, **41**, 217).

Lead workers whose recorded PbB levels had never exceeded 70 μg/dl (mean $\pm$ 1SD, 32 $\pm$ 11 μg/dl) also showed impaired psychological performance in 16 tests in comparison with a group of 24 control subjects matched with regard to sex, education, job quality and mean age. Moreover, even within the group of 49 lead workers there was a significant relationship between lead uptake and impaired performance, particularly in tests of visual intelligence and visual-motor functions. The correlation remained significant irrespective of whether maximum recorded, present or time-weighted average blood levels were used as a measure of uptake, although the last gave the strongest correlations. Almost all poor results belonged to subjects with a maximum PbB level above 50 μg/dl (Haenninen *et al. J. occup. Med.* 1978, **20**, 683).

Conclusions

It is evident that PbB levels prevalent in the general population are sufficient to produce a significant inhibition of ALAD activity, although this is not thought to be of biological significance. However they are also sufficient to increase EP levels, particularly in children, a finding of more potential concern. In addition there is some evidence that suggests that very low body burdens of lead (perhaps even at PbB levels below 15 μg/dl) may impair both the intelligence of children and their brain function as indicated by EEG data, the pattern of response apparently following a continuum rather than occurring above a definite threshold level. However, the significance of the EEG data in terms of IQ and behaviour is not clear. In the studies of the effects of lead on intelligence, behaviour and cerebral electrical activity, attempts to correct for socio-economic factors and for behaviour such as pica tend to reduce the statistical significance of these findings, although some degree of correlation still appears to remain. More careful control of such factors might totally abolish the relation with lead levels. However it would seem to be unwise to make this assumption on the present evidence from studies in man. We will consider next the results of studies in animals.

[C. Rostron—BIBRA]

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ABSTRACTS AND COMMENTS

Your favourite tipple: mutagenicity...

Nagao, M., Takahashi, Y., Wakabayashi, K. & Sugimura, T. (1981). Mutagenicity of alcoholic beverages. *Mutation Res.* **88**, 147.

Although recent well-publicized reports have indicated that the moderate consumption of beer or wine may offer some protection against cardiovascular disease (*Cited in F.C.T.* 1981, **19**, 789), other reports suggest a link between the consumption of certain alcoholic beverages and some forms of cancer (*ibid* 1981, **19**, 781). That such an association may exist is also indicated by the presence of *N*-nitrosamines in alcoholic beverages (Goff & Fine, *Fd Cosmet. Toxicol.* 1979, **17**, 569; Scanlan, Barbour, Hotchkiss & Libbey, *ibid* 1980, **18**, 27), an increased incidence of sister-chromatid exchanges in alcoholics (*Cited in F.C.T.* 1982, **20**, 237) and the mutagenicity of concentrates or fractions of alcoholic beverages in *in vitro* tests (Goff & Fine *loc. cit.*; *Cited in F.C.T.* 1981, **19**, 781; *ibid* 1982, **20**, 335). The paper cited above provides further data on the mutagenicity of certain alcoholic drinks.

Evaporated residues of 13 brands of whisky (six from Japan, four from Scotland, two from the USA and one from Canada), five brands of French brandy and a French apple brandy were tested. The tests were conducted on *Salmonella typhimurium* strain TA100 (a base-pair-change mutant) and TA98 (a frameshift mutagen), using a modification of the Ames method described by Nagao *et al.* (*Mutation Res.* 1977, **42**, 335). Evaporated residues of all of the samples of whisky except one from Scotland, all of the brandies and the apple brandy were mutagenic to TA100 in the absence of S-9 mix derived from the livers of PCB-treated rats. In the presence of S-9 mix the mutagenicity was decreased or abolished. Only one of the samples, a whisky, showed any mutagenicity towards TA98 and this was weaker than that towards TA100.

The components of the beverages that were responsible for the mutagenicity are not known, although the authors report that a preliminary experiment showed that brandy that had been distilled but not yet matured in the barrel was not mutagenic.

[This study obviously does not address the role ethanol itself may have in any genotoxic effects of alcoholic beverages. The use of different methods of deriving fractions of alcoholic beverages in mutagenicity tests makes it difficult to compare the results. In one study (Lee & Fong, *Fd Cosmet. Toxicol.* 1979, **17**, 575), using concentrates obtained by passage through XAD-2 chromatographic columns, no mutagens were detected in an Ames test of samples of alcoholic beverages imported into Hong Kong, although concentrates from Chinese alcoholic spirits did show mutagenic activity; some of the concentrates required metabolic activation and others did not. Loquet *et al.* (*Mutation Res.* 1981, **88**, 155) used distillation and evaporation to prepare aqueous, alcoholic and non-volatile fractions of alcoholic beverages. The alcoholic fraction of a sample of whisky was mutagenic in the presence of S-9 mix, but aqueous fractions of two samples of brandy were mutagenic in the absence of S-9 mix.]

...and foetal effects

Abel, E. L., Dintcheff, B. A. & Bush, R. (1981). Effects of beer, wine, whiskey, and ethanol on pregnant rats and their offspring. *Teratology* **23**, 217.

The adverse effects of excessive alcohol consumption on the foetus are well documented (*Cited in F.C.T.* 1980, **18**, 314) and some studies have indicated that moderate use of alcohol during pregnancy may affect the foetus and that the effects may vary with the type of beverage consumed (*ibid* 1982, **20**, 238). In the study cited above pregnant rats were incubated twice daily throughout gestation with beer, wine, whisky or 95% ethanol, all given at doses equivalent to 3 g ethanol/kg body weight. All of the animals were pair-fed to those receiving ethanol. One control group was given distilled water containing sucrose (isocaloric to the alcohol solutions used) and was pair-fed with the ethanol-treated group. Another control group was given food and water *ad lib.* The litters were culled, to five males and five females if possible, and each litter was nursed by a surrogate mother that had delivered within 48 hr of the actual mother but had been untreated during gestation.

Animals in each of the four alcohol-treated groups weighed significantly less than those in the pair-fed control group at birth and at 22 days of age. The pups exposed to alcohol *in utero* also showed impaired performance in a test of motor performance at 17 days of age. However, neither in these parameters nor in any others measured were there any significant differences between the pups from mothers treated with the different types of alcoholic beverages. Similarly there were no significant differences between the alcohol-treated groups in maternal blood-ethanol levels, pregnancy loss, length of gestation or maternal food or water consumption, and although whisky-treated dams did gain significantly less "adjusted" weight (maternal weight-litter weight) than the pair-fed controls, the difference between the whisky- and ethanol-treated groups was not significant. It is concluded that the congeners present in alcoholic beverages do not potentiate the effects of alcohol at least with regard to embryonic/foetal development of rats.

Reproduction studies with OPP

John, J. A., Murray, F. J., Rao, K. S. & Schwetz, B. A. (1981). Teratological evaluation of orthophenylphenol in rats. *Fundamental appl. Toxic.* **1**, 282.

Orthophenylphenol (OPP) is a powerful broad spectrum disinfectant and fungicide. Ingestion of OPP

residues by man may occur as a result of its post-harvest application to fruit and vegetables for preservation purposes. An acceptable daily intake of 1 mg OPP/kg body weight was recommended by FAO/WHO in 1969 but further work including a reproduction study in experimental animals was considered desirable (1969 Evaluations of Some Pesticide Residues in Food. FAO/PL: 1969/M/17/1. WHO/Food Add. 70.38). Since that time, reports of several reproduction studies have emanated from Japan. Shirasu *et al.* (*Mutation Res.* 1978, **54**, 227) reported that OPP given to mice at oral doses of 100 and 500 mg/kg/day for 5 days induced no dominant-lethal mutations at any stage of spermatogenesis. In addition, John *et al.* (*loc. cit.*) cite studies in which there was no evidence of teratogenic potential when pregnant mice were given 1450–2100 mg OPP/kg/day by mouth on days 7–15 of gestation or in Wistar rats receiving 150–1200 mg/kg/day on days 6–15 of pregnancy. A dose of 600 mg/kg/day was reported to be toxic to the Wistar dams and their foetuses. The present study carried out at the Dow Chemical Toxicology Research Laboratories, was intended to evaluate the embryotoxic potential of OPP in Sprague–Dawley rats following oral administration at a maximum tolerated dose.

Preliminary studies indicated that doses of 800 mg/kg/day and above were toxic to the dams, so for the main study doses of 100, 300 or 700 mg OPP/kg/day were administered by gavage to groups of 25–27 pregnant Sprague–Dawley rats on days 6–15 of gestation. A group of 35 controls received the vehicle, cottonseed oil. The rats were observed and weighed daily during the dosing period and their food and water consumption was measured at 3-day intervals. The rats were killed on day 21 of gestation and the numbers of live, dead and resorbed foetuses were recorded, along with foetal size and body weight and any signs of external alterations, cleft palate or skeletal alterations. One-third of the foetuses from each litter were selected at random and also examined for soft tissue alterations.

A minimal toxic effect on the pregnant rats in the high-dose group was noted and this was manifested in reduced food consumption and body-weight gain in early gestation, and a reduction in the mean liver weight. OPP had no effect on the number of implantation sites per dam, the mean litter size, the incidence of resorptions, or the foetal body measurements. No major malformations were observed among litters of rats given 100 or 700 mg OPP/kg/day although one foetus from a rat given 300 mg/kg/day had a hypoplastic tail. Statistically significant ($P < 0.05$) increases in the incidences of delayed ossification of sternebrae and foramina (pinpoint holes) in the skull among litters in the high-dose group were not considered to be indicative of teratogenicity. Both were considered to be minor skeletal variants and have been observed historically at low incidences among litters from untreated rats. A single occurrence of an extra hemivertebra and rib was observed in a foetus from the low-dose group. These results support earlier findings of a lack of teratogenic potential in rats and show that there were no embryotoxic effects at doses of up to 700 mg OPP/kg body weight/day in Sprague–Dawley rats.

Hormonal effects of carbon disulphide

Wägėr, G., Tolonen, M., Stenman, U.-H. & Helpiö, E. (1981). Endocrinologic studies in men exposed occupationally to carbon disulphide. *J. Toxicol. envir. Hlth* **7**, 363.

Exposure to carbon disulphide (CS_2) has been linked with a number of toxic effects including coronary heart disease, retinopathy, peripheral neuropathy, diabetes and disturbances in glucose metabolism (*Cited in F.C.T.* 1978, **16**, 494; *ibid* 1979, **17**, 685). The present study concerns the effect of prolonged exposure to CS_2 on certain hormones.

Fifteen men exposed to CS_2 at a viscose rayon plant in Finland for 10–36 yr (mean 23 yr) were compared with 16 age-matched employees at a paper mill in the same town. The viscose rayon factory had been operating since the early 1940s and CS_2 and H_2S concentrations in the workroom air had been monitored regularly. The mean concentrations of CS_2 and H_2S were 'very high' in the 1940s, *c.* 60–120 mg/m^3 in the 1950s, *c.* 30–90 mg/m^3 in the 1960s and less than 30 mg/m^3 in the 1970s. Furthermore since the early 1970s the daily duration of exposure had been shortened considerably as ventilated surveillance rooms had been constructed in the spinning department. Both groups of workers were given a routine clinical examination and their medical histories were recorded. Blood specimens for hormone determinations were drawn in the morning after an overnight fast. The blood was analysed for total serum thyroxine (T_4), total serum triodothyronine and thyrotropin. The binding of T_4 to serum proteins (T_4U) was also determined and the free thyroxine index (FTI) was calculated ($FTI = T_4 \times T_4U$). In addition, the thyrotropin releasing hormone (TRH) stimulation test was carried out by administering 200 μg synthetic TRH iv and measuring serum thyrotropin levels 0, 20 and 60 min later. Levels of serum testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (before and after TRH stimulation) and basal serum cortisol were also measured.

No differences were observed between the two groups of workers in levels of thyroid hormones, cortisol, prolactin and testosterone. However, both FSH and LH levels were higher in the group exposed to CS_2 than in the men from the paper mill, although there was no good correlation between elevated FSH or LH and duration of exposure. In seven of the cases studied, values of FSH or LH were above the set reference limit. Ten of the 15 exposed men reported some degree of past or present sexual impotence whereas none of the controls admitted to this problem. No individual correlation was observed between reported impotence and hormone levels.

In previous studies a decrease in serum T_4 concentration was observed in workers exposed to CS_2 (Cavalleri, *Archs envir. Hlth* 1975, **30**, 85), but no effects on thyroid function were observed in the present study. The normal responses of thyrotropin and prolactin to TRH indicate that hypothalamic–pituitary function was normal, and the raised LH and FSH levels are attributed to testicular damage, since testosterone levels were normal, they are taken as signs of latent primary gonadal insufficiency. These

effects may reflect high exposure during past decades rather than the men's current exposure to low CS_2 concentrations.

Fishy allergy?

Droszcz, W., Kowalski, J., Piotrowska, B., Pawlowicz, A. & Pietruszewska, E. (1981). Allergy to fish in fish meal factory workers. *Int. Archs occup. envir. Hlth* **49**, 13.

Fish is said to be a frequent cause of food allergy, reactions generally resulting from ingestion. However, nearly 40% of workers in a factory where they were exposed to an aerosol of prawn generated during processing developed wheezing and other respiratory symptoms (Gaddie *et al. Lancet* 1980, **II**, 1350). In tests, 24% of the workers had positive skin tests for prawn and 62% had serum precipitins.

The present study has a similar theme and concerns an investigation of fifty-one workers in fish meal factories in four Polish harbour cities. Lengths of employment in the factories varied from 1 to 33 yr, with an average of 10·7 yr and 44 of the workers examined were smokers. Each of the workers completed a questionnaire about medical history and was given a chest X-ray, physical examination and tests to assess respiratory function. Additionally, skin tests were carried out with 12 common allergens and intradermal and nasal provocation tests were conducted with ten fish genera. Total serum IgE and serum precipitins to fish antigens were determined. Twenty-nine of the workers exhibited symptoms of chronic bronchitis. Twelve of them showed a positive response to fish allergens in skin tests, and these included the three who reported a clinical allergy to fish (two cutaneous and one respiratory). The two workers with a history of cutaneous allergy to fish had elevated serum IgE levels. None of the workers had serum precipitins against fish antigens. Most of the respiratory changes observed could be attributed to smoking habits and none could be directly related to fish processing.

It is concluded that none of the workers had symptoms of allergic alveolitis induced by fish protein exposure and that clinical allergy confirmed by immunological tests was found in only two subjects. Presumably the positive reactions in skin tests using fish allergens have little clinical significance in most of the workers. It seems that occupational exposure to fish meal does not pose a significant risk of developing allergic reactions.

Quartz in coal-mines—time for concern

Seaton, A., Dick, J. A., Dodgson, J. & Jacobsen, M. (1981). Quartz and pneumoconiosis in coalminers. *Lancet* **II**, 1272.

It was assumed that pneumoconiosis in coal-miners was caused by the quartz present in airborne coal-mine dust until Collis & Gilchrist (*J. ind. Hyg. Toxicol.* 1928, **10**, 101) showed that very similar lesions occurred in coal-trimmers exposed to very little free silica. A major survey of British coal-mines (Jacobsen *et al. Nature, Lond.* 1970, **227**, 445) showed that the risk of developing pneumoconiosis was dependent on total dust exposure and did not correlate with the quartz content of the dust. The quartz levels in the dusts encountered were 0·8–7·8%. Walton *et al.* (In *Inhaled Particles IV*. Part 2. Edited by W. H. Walton, p. 669. Pergamon Press, Oxford, 1975) who further analysed the individual data on the populations studied by Jacobsen *et al.* similarly concluded that the total concentration of respirable dust was the most suitable index of the dust hazard where the quartz content of the dust was below 7·5%. The present paper indicates that quartz may after all play an important role in the development of pneumoconiosis.

During a routine radiological survey in 1978, 623 miners in a Scottish colliery were examined and the chest radiographs of 21 of the men, an unusually high number, showed progression of simple pneumoconiosis when compared with radiographs taken 50 months earlier. A second, independent, reading of these 21 cases and matched controls confirmed that at least 18 of the identified men had definite signs of progression, the remaining three being borderline. At this colliery observations including regular radiographical surveys and measurements of cumulative life-time exposures of all of the men to mixed coal-mine dust, and of the quartz component of the dust had taken place since 1954. Comparing the dust and quartz exposure for the 21 cases and matched controls, over the 50 months since the last radiographs had been taken, showed that the affected men had been exposed to a significantly higher level of mixed dust and to a significantly higher proportion of quartz in the mixed dust—12·98% quartz compared with 8·44% in the controls. The cases were exposed to a significantly lower level of coal in the mixed dust than were controls but there was no difference in the levels of non-quartz minerals to which the two groups were exposed. No pattern could be observed when the individual life-time exposures of cases and controls were compared; three men developed pneumoconiosis after cumulative exposures to mean quartz concentrations of less than 0·1 mg/m^3.

Thus an unexpectedly rapid progression of pneumoconiosis was observed in some cases even after generally low exposures to mixed coal-mine dust, and this occurred where the proportions of quartz in the mixed dust had been unusually high since 1974 (13 and 8% of the dust for the cases and controls respectively). The authors concluded that quartz must be regarded as a possible cause of pneumoconiosis in miners particularly where mixed dust exposures were low but the proportion of quartz present was high. The finding that there was a high risk of disease in men with cumulative exposure levels above 0·1 mg quartz/m^3 and some risk in men with even lower exposures should be of considerable use in defining control limits for quartz exposure both in coal-mining and in other dusty industries.

Acute and subacute iodoform inhalation

Tansy, M. F., Werley, M., Landin, W., Oberly, R., Kendall, F. M., Miller, A. & Sherman, W. (1981).

Subacute inhalation toxicity testing with iodoform vapour. *J. Toxicol. envir. Hlth* **8**, 59.

Iodoform has been in widespread use for many years and it has been studied in a number of tests, usually involving oral or subcutaneous administration (Kutob & Plaa, *Toxic. appl. Pharmac.* 1962, **4**, 354; Reynolds, *Biochem. Pharmac.* 1972, **21**, 2555), including an NCI bioassay in rats and mice (*Federal Register* 1978, **43**, 46382). The latter study provided no "convincing evidence" of carcinogenicity of iodoform given by gavage to male rats at 142 or 71 mg/kg body weight/day, female rats at 55 or 27 mg/kg/day or male or female mice at 93 or 47 mg/kg/day, respectively, on 5 days/wk for 78 wk. The study reported now is the first to provide data on the toxicity of iodoform vapour.

Groups of five male and five female Sprague–Dawley rats were exposed to 0, 140, 170 or 230 ppm iodoform for 7 hr and then observed for 48 hr in order to determine the LC_{50} value. Other groups of five male and five female rats were exposed to 0, 1 or 14 ppm for 7 hr/day for 7 days to assess the subacute effects of iodoform. The animals in the subacute study were separated after each exposure and housed in individual metabolism cages for the subsequent 17 hr. They were weighed daily, food and water consumption were measured, and urine and faeces were collected. Metabolic studies were also carried out for 17 hr/day for 1 wk before and 1 wk after the week of iodoform exposure. At the end of this period, intestinal transit time was measured, blood samples were taken for analysis and some of the rats were killed and autopsied.

Following acute exposure, the animals were seen to huddle, close their eyes and breathe rapidly and shallowly. Imminent death could be predicted by an obvious blanching of the ears, which was attributed to general cardiopulmonary collapse. The calculated 7-hr LC_{50} value was 183 ppm when based on mortality over 24 hr, but fell to 165 ppm on the basis of 48-hr mortality, indicating delayed death after iodoform exposure.

In the subacute study, no significant differences were noted in food and water intake, urine and faeces output or intestinal transit time. There were statistically significant differences between the treated groups and the controls in some blood chemistry values, but the majority of these values still fell within the normal range or the differences were not dose-related. Histopathological examination of tissues taken at autopsy revealed no treatment-related changes except for large mineral deposits in the renal medullary tubules of the rats in the 14-ppm group, and in this group there was also some cloudy swelling of the liver cells.

[It is unfortunate that some higher doses of iodoform were not tested in the subacute study.]

Metabolism of bisphenol A epoxy

Climie, I. J. G., Hutson, D. H. & Stoydin, G. (1981). Metabolism of the epoxy resin component 2,2-bis[4-(2,3-epoxypropoxy)phenyl]propane, the diglycidyl ether of bisphenol A (DGEBPA) in the mouse. Part I. A comparison of the fate of a single dermal application and of a single oral dose of ^{14}C-DGEBPA. Part II. Identification of metabolites in urine and faeces following a single oral dose of ^{14}C-DGEBPA. *Xenobiotica* **11**, 391 & 401.

To the toxicologist, the epoxy moiety is associated with very dark images. Many epoxies are direct-acting alkylating agents and are likely to pose a carcinogenic or mutagenic threat to man. It must, therefore, have come as a pleasant surprise to the polymer industry when the diglycidyl ether of bisphenol A (DGEBPA), and the high-tonnage resins of which it is a major component, demonstrated only weak genotoxic activity in the initial phase of toxicity testing (*Cited in F.C.T.* 1979, **17**, 420; *ibid* 1980, **18**, 444). Metabolic studies have now been conducted that go some way to explaining the apparent reluctance of DGEBPA to live up to its structural promise.

Single doses of 56 mg [^{14}C]DGEBPA/kg body weight applied to the skin of male CF1 mice were found to be only slowly absorbed. One day after treatment, 90% of the radioactivity could still be extracted from the site of application or its covering foil, and most (97%) of that on the skin was unchanged. Even at day 8, some 40% was still recoverable locally (81% unchanged). The maximum excretion of radioactivity occurred on day 2, when the 24-hr urine contained 1·3% and the faeces 8% of the dose. In contrast, excretion of the label was rapid following oral administration. Almost 90% of a single dose of 55 mg/kg body weight given by gavage was eliminated by day 2, predominantly (about 80% of the dose) in the faeces.

Despite the very different elimination rates following dermal and oral administration, preliminary analysis by thin-layer chromatography showed that the profile of urinary and faecal metabolites of systemically absorbed DGEBPA was independent of the route of administration. Further elucidation of the metabolic pathways was therefore limited to animals treated orally. The major metabolite, accounting for 24% of an administered dose of about 55 mg/kg body weight, was 2-[4-(2,3-dihydroxypropoxy)phenyl]-2-[4′-(2-carboxy-2-hydroxyethoxy)phenyl]propane formed, presumably, by hydrolytic opening of the two epoxide groups and oxidation of one of the two diol moieties to give the α-hydroxycarbocylic acid. Decarboxylation of this compound followed by oxidation was thought to be the source of the other major metabolite, identified as 2-[4-(2,3-dihydroxypropoxy)-phenyl]-2-[4′-(carboxymethoxy)phenyl]propane, which accounted for 14% of the administered dose.

Of the minor metabolites, there were three of particular interest. Two of these were related to the major metabolites, the 2,3-dihydroxypropoxy moiety of the diol acid and of the diol α-hydroxyacid being replaced by a 3-methylsulphonyl-2-hydroxypropoxy side chain. They were the only direct evidence of a possible alkylating action by DGEBPA; it was suspected that they were derived from an attack on an electrophilic centre, probably the epoxide. However, since the yields of each were small, only 5 and 4% of the dose, only weak alkylating ability was indicated. The third minor metabolite of mechanistic interest,

accounting for some 5% of the dose, was the phenoldiol of DGEBPA, 2-[4-(2,3-dihydroxypropoxy)phenyl]-2-(4'-hydroxyphenyl)propane. Of the two pathways likely to be involved in its formation, one would generate glycidaldehyde, an Ames-positive compound and a skin carcinogen of possible importance as a component of DGEBPA's weak genotoxicity. The other likely pathway would liberate glyceraldehyde, an endogenous compound, presumably of low toxic potential. Unfortunately, the best way of differentiating between these two routes—the synthesis of DGEBPA labelled in the detaching 3-carbon group—was not a practical possibility, so some clues were sought in a series of *in vitro* studies. However, oxidative dealkylation of DGEBPA by mouse-liver microsomal monooxygenase could not be detected *in vitro*; the epoxide-hydratase reaction (opening the epoxide bonds with formation of the bisdiol) was shown to proceed very rapidly. Only in the presence of an epoxide-hydratase inhibitor could a 1% conversion to the phenoldiol be demonstrated, and the major product under these conditions was the epoxide diol. The investigators considered that a high epoxide-hydratase activity would be likely to lead to rapid conversion of DGEBPA to the bisdiol *in vivo* with subsequent oxidative reactions yielding carboxylic acids and phenols, a sequence liberating glyceraldehyde rather than the glycidaldehyde. The fact that 3% of an orally administered dose of the bisdiol was eliminated as the phenoldiol, a yield comparable to the 5% seen from DGEBPA itself, supported the view that the bisdiol could undergo oxidative dealkylation to the phenoldiol *in vivo* although this could not be demonstrated *in vitro*. It was concluded, therefore, that the more direct conversion of DGEBPA to the phenoldiol, a reaction that would lead to the liberation of glycidaldehyde, probably did not occur to any measurable extent in the intact mammal—or indeed *in vitro*, at least in the absence of competitive inhibition of epoxide hydratase. DGEBPA has been shown to react with glutathione, in the presence of a glutathione-epoxide transferase taken from a mouse-liver cytosol fraction, to form a polar conjugate. The bisdiol, however, was not a substrate for this enzyme. No evidence could be found *in vivo* for the formation of this glutathione conjugate, providing further support for the predominance of the epoxide-hydratase pathway and the *in vivo* formation of glyceraldehyde rather than glycidaldehyde.

The apparent importance of the epoxide hydratase in minimizing the mutagenic potential of DGEBPA led to an interspecies comparison of total hepatic DGEBPA epoxide hydratases. The activities determined in the livers of rabbits, mice and rats were in the ratio of 3:2:1. In the mouse a large amount of this enzyme activity was due to a soluble form present in the cytosol fraction of the liver; previously reported work with other species (using other epoxide substrates) had shown low levels of soluble hydratases, the membrane-bound enzymes being far more important components of total activity. It is still too early to be confident of man's ability to cope with the epoxide moiety. Work on styrene oxide (Oesch *et al. Biochem. Pharmac.* 1974, **23**, 1307) suggests that if styrene oxide is typical of other substrates, the liver of man possesses a relatively high hydratase activity compared with that of other mammals, higher indeed than that of the rabbit. Man also seems to rate fairly highly in skin hydratase activity, but, as in other animals, the enzyme activity in the skin is far lower than that found in the liver.

Another look at the foetal effects of MEK

Deacon, M. M., Pilny, M. D., John, J. A., Schwetz, B. A., Murray, F. J., Yakel, H. O. & Kuna, R. A. (1981). Embryo- and fetotoxicity of inhaled methyl ethyl ketone in rats. *Toxic. appl. Pharmac.* **59**, 620.

In 1974 we reported a study of the effects of *in utero* exposure of Sprague–Dawley rats to 1000 or 3000 ppm methyl ethyl ketone (MEK; *Cited in F.C.T.* 1975, **13**, 481). Among the offspring of rats inhaling the higher dose of MEK, 7 hr/day on days 6–15 of pregnancy, there was a low incidence of true foetal abnormalities (acaudia, imperforate anus and brachygnathia), and a significantly increased incidence of soft-tissue malformations and sternebral variations and at the lower level, there was a significant increase in the total number of litters containing foetuses with skeletal anomalies. No maternal toxicity was reported. This study seems to have been widely accepted as evidence of the embryotoxic, foetotoxic and potentially teratogenic effects of MEK exposure and little work has since been undertaken on this aspect of MEK exposure.

However, the experiment has now been repeated by the same group of workers from the Dow Chemical Toxicology Research Laboratories. Only one additional dose level, 400 ppm, has been used and in most other respects this study is identical in methodology to the former. The results are rather different from those obtained earlier. Slight maternal toxicity (decreased weight gain and increased water consumption) occurred in rats inhaling 3000 ppm MEK, but there was no evidence of embryotoxicity or teratogenicity at any dose level. At the high dose level a significant increase in some minor skeletal variations indicated a slight foetotoxic effect.

[The results of the two studies indicate that *in utero* exposure to MEK may cause skeletal variations in rats, but whether other adverse effects are induced seems to be a question that can only be resolved by further research.]

Retinyl acetate and mouse mammary tumours

Welsch, C. W., Goodrich-Smith, M., Brown, C. K. & Crowe, N. (1981). Enhancement by retinyl acetate of hormone-induced mammary tumorigenesis in female GR/A mice. *J. natn. Cancer Inst.* **67**, 935.

During recent years, evidence from both experimental and clinical investigations has been accumulating to suggest that vitamin A and its derivatives, the retinoids, possess anti-carcinogenic effects (*Cited in F.C.T.* 1982, **20**, 333). The finding that retinoids could inhibit the emergence of bladder carcinomas in carcinogen-treated rats (Sporn *et al. Science, N.Y.* 1977, **195**, 487) has led to the testing of certain reti-

noids in patients at high risk for bladder cancer (Sporn, *Nature, Lond.* 1978, **272,** 402). The antineoplastic effects of retinoids in rats have also been demonstrated against carcinogen-induced cancers of the colon (Newberne & Suphakarn, *Cancer, N.Y.* 1977, **40,** 2553), respiratory tract (Cone & Nettesheim, *J. natn. Cancer Inst.* 1973, **50,** 1599) and mammary gland (Moon *et al. Cancer Res.* 1976, **36,** 2626), and in mice experimentally induced skin and bladder tumours are also susceptible to retinoid therapy (Becci *et al. Cancer Res.* 1978, **38,** 4463; Bollag, *Eur. J. Cancer* 1974, **10,** 731).

However, several experimental studies have not confirmed the antitumour properties of retinoids (Schroder & Black, *J. natn. Cancer Inst.* 1980, **65,** 671) and it has now been demonstrated that retinyl acetate enhances, rather than inhibits, the carcinogenic process when tested in a hormonally induced mammary cancer model in the mouse.

Virgin and ex-breeder female GR mice were given oestrone (0·5 mg/litre drinking-water) and progesterone (30-mg pellet administered subcutaneously once a month) for 13–14 wk. Half the mice were fed retinyl acetate (82 mg/kg diet, the maximally tolerated dose) throughout the study, and the animals were examined for mammary tumour development (experiment 1). The experiment was subsequently repeated (experiment 2), using a 1–2 wk longer treatment period and older multiparous mice.

In the first experiment, the mammary carcinoma incidence in nulliparous control and retinoid-treated mice was 22/65 (34%) and 37/65 (57%) respectively ($P < 0{\cdot}05$). In experiment 2, the percentages in groups of 48 mice were 56 and 77% respectively ($P < 0{\cdot}05$). The total numbers of mammary tumours were increased in both experiments. Multiparous mice fed control and retinoid diets developed mammary carcinomas in 13/30 (43%) and 23/30 (77%) mice respectively ($P < 0{\cdot}05$) in experiment 1, but in the repeated experiment all multiparous mice (19/19 and 19/19 respectively) developed mammary cancer. The authors explain the overall increase in mammary tumours in experiment 2 in terms of mouse ages and longer treatment periods, and speculate that retinoids may enhance mammary neoplasia by increasing the sensitivity of mouse tissues to hormones. The findings of increased mammary gland development, and uterine and adrenal weights after retinoid treatment are in keeping with this suggestion.

This report is in contrast to previous observations from the same laboratory in which retinyl acetate induced a profound inhibition of *N*-methyl-*N*-nitrosourea-induced mammary cancer in Sprague–Dawley rats (Welsch *et al. Cancer Res.* 1980, **40,** 3095) and also differs from the only other evaluation of retinoid activity on mouse mammary neoplasia, in which retinyl acetate had no effect on spontaneous mammary cancer in C3H/A mice (Maiorana & Gullino, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1979, **38,** 1450).

[Both spontaneous and experimentally induced mammary cancer in rats and mice contrast markedly. Viruses are implicated in the latter species and the morphogenesis of tumours in each species is different. However until the activity of the retinoids against both carcinogen- and hormonally induced tumours is compared in both the rat and the mouse, the relative significance of the species and of the inducing agent involved cannot be assessed. Nevertheless, since hormonal factors play a significant, albeit poorly understood, part in human breast cancer, the findings of this study should caution against the premature clinical use of retinoids.]

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LETTERS TO THE EDITOR

TOXICITY OF VERSALIDE

Sir.—Butterworth and Mason have recently reported in your publication (*Fd Cosmet. Toxicol.* 1981, **19**, 753) on the acute oral toxicity of Versalide®. In their paper they state that "there appear to be no readily available data on the safety-in-use..." of Versalide, which has also been described as acetylethyltetramethyltetralin (AETT). In actual fact, a rather extensive fragrance monograph has been published in this very journal (*ibid* 1979, **17**, 357), describing in summary fashion the work carried out on this compound in our laboratories. The phenomena of blue tissue discoloration and cumulative neurotoxicity were first noted by us in 1975, and subsequent publications described the neuropathy in detail (Spencer *et al. Neurotoxicology* 1979, **1**, 221; *idem, Science, N.Y.* 1979, **204**, 633).

I strongly encourage the authors to read these and other published reports relating to the neurotoxicological potential of AETT, and to incorporate these references into future bibliographies offered in support of new research into this fascinating chemical.

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PASSIVE SMOKING: NICOTINE, A HAPTEN

Sir,—In his Review Article on passive smoking (*Fd Chem. Toxic.* 1982, **20**, 223) P. N. Lee mentioned the subject of allergy and tobacco smoke and commented that "whether this was a psychological, rather than a physiological, response is open to question". My recent study about the haptenic rôle of the nicotine alkaloid of *Nicotiana tabacum* in the aetiology of a dermatitis in my family (Sudan, *Allergie Immunol.* 1978, **10**, 36; Sudan & Sterboul, *Nouv. Presse méd.* 1979, **8**, 3563) was not considered. This dermatitis resembles 'seborrhoeic dermatitis'.

In all the patients, the *in vitro* human basophil degranulation test (HBDT) of Benveniste (*Clin. Allergy* 1981, **11**, 1) gave positive results with either the allergen, tobacco (leaves, Hollister-Stier: >50% from 10 μg to 10^{-1} μg/ml) or the hapten, nicotine (>50% from 1 μg to 10^{-2} μg/ml) bound to human serum albumin (HSA). RAST (radioallergosorbent test) results were positive for myself with allergenic extracts of tobacco leaves: 4 + (tobacco leaf A), 3 + (tobacco leaf B) and 3 + (tobacco leaf C) (S. B. Lehrer, New Orleans, USA). These results were in accord with a familial and personal history of atopic reactions and suggested that the skin mast cells (by virtue of their capacity to release the chemical mediators of anaphylaxis) were the cause of the clinical symptoms. Topical sodium chromoglycate seems to be of benefit in this reaction (Sudan & Sterboul, *Cutis, Paris* 1980, **4**, 81) and the specific desensitization with allergenic extracts of tobacco leaves (Hollister-Stier) is very satisfactory.

We have recently studied a new case of sensitization to the nicotine in a patient afflicted with a seborrhoeic dermatitis (Sudan & Sterboul, *Br. J. Derm.* 1981, **104**, 349). The HBDT test showed strongly positive results with the allergens, tobacco (leaves, Hollister-Stier: 66%, 100 μg/ml) and nicotine–HSA (58%, 10 μg/ml).

Avoidance of tobacco smoke has cleared the dermatitis in all these patients. Thus there appears to be a cellular reaction to tobacco antigens detectable in a syndrome of unknown aetiology classified for a century as 'seborrhoeic dermatitis' (Unna, *Mh. prakt. Derm.* 1887, **6**, 827).

Contrary to the conclusion that "there seems at present to be no convincing evidence that passive smoking results in any material risk of serious disease for the healthy nonsmoker" (Lee, *loc. cit.*), our study demonstrates that "a single puff of tobacco smoke can induce an anaphylactic reaction of the skin" of passive smokers (Sudan, *Br. med. J.* 1978, **2**, 895) in some circumstances.

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MEETING ANNOUNCEMENTS

DIET AND NUTRITION CONFERENCE

An international conference on diet and nutrition is to be held in Tel Aviv, Israel on 21–23 February 1983. The Conference will cover a broad range of topics including for example the influence of food on sleep, diet and blood lipids, nutritional status and guar gum. Further details may be obtained from The Secretariat, International Conference on Diet and Nutrition, P.O. Box 29784, Tel Aviv 61297, Israel.

CELL BIOCHEMISTRY AND FUNCTION MEETING

The "1st International Meeting on Cell Biochemistry and Function" is to be held at the University of Surrey on 23–25 March 1983. Further details may be obtained from Professor J. W. Bridges, Secretary, '1st International Meeting on Cell Biochemistry and Function' Scientific Committee, Institute of Industrial and Environmental Health and Safety, University of Surrey, Guildford, Surrey, UK, GU2 5XH.

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FORTHCOMING PAPERS

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

Effect of dietary butylated hydroxyanisole on methylazoxymethanol acetate-induced toxicity in mice. By B. S. Reddy, K. Furuya, D. Hanson, J. DiBello and B. Berke.

Lack of carcinogenicity of butylated hydroxytoluene on long-term administration to $B6C3F_1$ mice. By T. Shirai, A. Hagiwara, Y. Kurata, M. Shibata, S. Fukushima and N. Ito.

An investigation of the genetic toxicology of irradiated foodstuffs using short-term test systems. III—*In vivo* tests in small rodents and in *Drosophila melanogaster*. By H. W. Renner, U. Graf, F. E. Würgler, H. Altmann, J. C. Asquith and P. S. Elias.

Mutagenic effects of irradiated glucose in *Drosophila melanogaster*. By M. B. Varma, K. P. Rao, S. D. Nandan and M. S. Rao.

No volatile *N*-nitrosamines detected in blood and urine from patients ingesting daily large amounts of ammonium nitrate. By G. Ellen, P. L. Schuller, P. G. A. M. Froeling and E. Bruijns.

The influence of food flavonoids on the activity of some hepatic microsomal monooxygenases in rats. By M. H. Siess and M. F. Vernevaut.

Inactivation of aflatoxin B_1 mutagenicity by thiols. By M. Friedman, C. M. Wehr, J. E. Schade and J. T. MacGregor.

The effects of patulin and patulin-cysteine mixtures on DNA synthesis and sister-chromatid exchange frequency in human lymphocytes. By R. Cooray, K.-H. Kiessling and K. Lindahl-Kiessling.

The subchronic toxicity and teratogenicity of alternariol monomethyl ether produced by *Alternaria solani*. By G. A. Pollock, C. E. DiSabatino, R. C. Heimsch and D. R. Hilbelink.

Occurrence, stability and decomposition of β-*N*[γ-L(+)-glutamyl]-4-hydroxymethylphenylhydrazine (agaritine), from the mushroom *Agaricus bisporus*. By A. E. Ross, D. L. Nagel and B. Toth.

Comparative toxicity of alkyltin and estertin stabilizers. By A. H. Penninks and W. Seinen.

Enzyme-mediated mutagenicity in *Salmonella typhimurium* of contaminants of synthetic indigo products. By W. M. F. Jongen and G. M. Alink.

Percutaneous absorption of 2-amino-4-nitrophenol in the rat. By H. Hofer, G. W. Schwach and Ch. Fenzl.

Reversibility of nephrotoxicity induced in rats by nitrilotriacetate in subchronic feeding studies. By M. C. Myers, R. L. Kanerva, C. L. Alden and R. L. Anderson.

Reversibility of renal cortical lesions induced in rats by high doses of nitrilotriacetate in chronic feeding studies. By C. L. Alden and R. L. Kanerva.

Estimation of volatile *N*-nitrosamines in rubber nipples for babies' bottles. By D. C. Havery and T. Fazio.

The mobilization of aluminium from three brands of chewing gum. By A. Lione and J. C. Smith. (Short paper)

The effect of dose and vehicle on early tissue damage and regenerative activity after chloroform administration to mice. By D. H. Moore, L. F. Chasseaud, S. K. Majeed, D. E. Prentice, F. J. C. Roe and N. J. Van Abbé. (Short paper)

The applicability of the draft EEC method for the determination of the global migration of plastics constituents into fatty food simulants to lacquers, plastics and laminates. By D. Van Battum, M. A. H. Rijk, R. Verspoor and L. Rossi. (Review paper)

CODATA BULLETIN

Editor: P S GLAESER, *CODATA Secretariat, Paris, France*

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S SUZUKI, On the use of a data base system for information processing in plant breeding.
Y MIYASHITA *et al,* The use of cluster analysis and display method of pattern recognition in structure-activity studies of antibiotics.
A R H COLE, Wavenumber standards in the infrared.
S R HELLER, & G W A MILNE, The NIH-EPA Chemical Information System (CIS).
P K BASU *et al,* Retrieval and simulation of UV photoelectron spectra of organic molecules.

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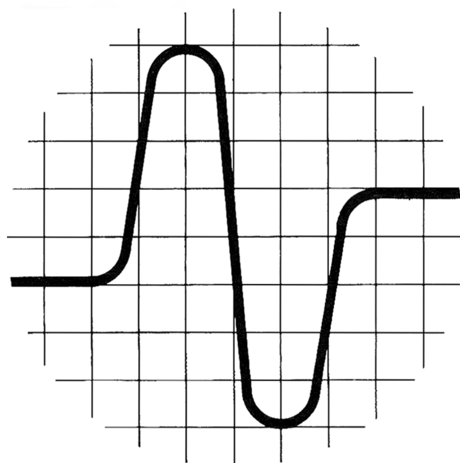

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