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Research Section

EFFECTS OF FD & C RED NO. 40 ON RAT INTRAUTERINE DEVELOPMENT

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(*Received 4 March 1980*)

Abstract—The food colouring FD & C Red No. 40 was tested for embryotoxicity and teratogenicity in Osborne–Mendel rats. The colouring was given throughout gestation either by oral intubation at levels of 7·5, 15, 30, 100 or 200 mg/kg body weight/day or as a 0·2% solution (260·2 mg/kg body weight/day) in the drinking water. No embryotoxic or foetotoxic (including teratogenic) effects were observed that could be related to exposure to FD & C Red No. 40 by either route of administration.

INTRODUCTION

FD & C Red No. 40 (Allura Red AC; C.I. 16035) was approved for use in food and drugs in 1971 (*Federal Register* 1971, **36**, 6892) but because of the wide acceptability of FD & C Red No. 2, FD & C Red No. 40 had only limited use as a food colouring until, in 1976, FD & C Red No. 2 was delisted (removed from the market place in the USA by regulatory action). From that time, the use of FD & C Red No. 40 expanded dramatically. By the end of 1978, it was the most widely used of the certified colourings. During 1978, nearly 1·8 million pounds of the colouring were certified by the Food and Drug Administration (FDA). FD & C Red No. 40 is also listed permanently for use in cosmetics (*Federal Register* 1974, **39**, 28278).

Only limited reproductive physiology data are available on FD & C Red No. 40. A two-generation study, with two litters per generation, was carried out on rats given doses of 0·37, 1·39 and 5·19% in the diet (Allied Chemical Corp., unpublished data 1970, in FDA files). The investigators reported that the dye caused decreased growth of the pups at the 5·19% dose level, but that no compound-related embryonic or teratogenic responses were found. The teratogenic potential of the dye was studied in albino rabbits by oral intubation of 0, 200 or 700 mg/kg on days 6–18 of gestation (Allied Chemical Corp., unpublished data 1974, in FDA files). When the animals were killed on day 29 of gestation, no indications of compound-related effects were apparent in either the maternal does or the developing foetuses.

After investigations on FD & C Red No. 2 (Collins & McLaughlin, 1972), a limited pilot study was carried out in our laboratory to test the embryotoxic and teratogenic potential of FD & C Red No. 40 in rats (T.F.X. Collins, unpublished data, 1974; summarized in *WHO Food Additive Series 1974 No. 6*, p. 39, WHO, Geneva, 1975). The present study was designed to provide more definitive data on the embryotoxic and teratogenic potential of FD & C Red No. 40 and, if either was demonstrated, to provide a basis for comparing the effects of administering the dye by gavage and in the drinking water.

EXPERIMENTAL

Material and animals. FD & C Red No. 40 is principally the disodium salt of 6-hydroxy-5-[(2-methoxy-5-methyl-4-sulphophenyl)azo]-2-naphthalene sulphonic acid. The dye, obtained from Allied Chemical Corp., Morristown, NJ (Lot no. Y9686), was assayed by the Certification Branch of the Division of Color Technology at the FDA and was found to consist of 88% FD & C Red No. 40, 5·3% NaCl, 4·5% volatile matter, 0·3% Na_2SO_4 and 0·26% Schaeffer's salt (an intermediate). The dye solutions were found to be stable for the duration of the experiment. Young adult virgin female Osborne–Mendel rats were obtained from the FDA breeding colony. Each experimental and control group contained at least 29 females. At mating, the females weighed 190–260 g (220 ± 14·6 g, mean ± SD). All of the animals were fed Purina Laboratory Chow (Ralston–Purina Co., Inc., St. Louis, MO) and were given distilled water *ad lib.* The animals were housed in stainless-steel hanging cages and the light cycle provided 12 hr darkness and 12 hr light.

Procedure. Females were mated with young adult Osborne–Mendel males at approximately 4.30 p.m. Copulation was confirmed by the presence of sperm in the vaginal lavage the following morning. Within each group a male impregnated a maximum of two females. Mated females were randomly allocated to one of the experimental or control groups. The day of finding sperm in the vaginal lavage was designated day 0 of gestation.

Solutions of FD & C Red No. 40 for both the intubation and drinking-water studies were prepared with distilled water. The rats were either intubated with 7·5, 15, 30, 100 or 200 mg FD & C Red No. 40/kg body weight daily on days 0–19 of gestation, or

were given the dye at 0·2% in the drinking water on days 0–20 of gestation. Concurrent control groups were intubated with distilled water or were given distilled water to drink. The intubation dose levels were the same as those used in the teratology study of FD & C Red No. 2 (Collins & McLaughlin, 1972), and the 0·2% solution was selected in order to provide information on the effects of the compound when ingested in small amounts *via* the drinking water. Based on average daily water consumption, it was calculated that the amount of dye ingested in the drinking water would be similar to that consumed at the highest dose level by intubation. The animals were intubated at the same time each day by the same personnel (whenever possible), and a 15-gauge needle with a smooth bead soldered onto the end was used to administer 1 ml/100 g body weight. For the drinking-water study, the 0·2% solution or distilled water was poured into glass bottles fitted with ATCO (Acto Manufacturing Co., Napa, CA) stainless-steel ball-point, non-drip tubes and was provided *ad lib.* from day 0 until the animals were killed on day 20; the fluid consumption of the animals in both groups was measured daily. All of the animals were weighed daily.

On day 20 of gestation, the animals were killed by CO_2 asphyxiation. The uterus was opened and examined *in situ* for the presence and location of resorption sites and live and dead foetuses. Deciduomas, brownish implantation sites without placentas, were called early deaths, and implantation sites with placentas and with complete but non-viable foetuses that were of subnormal size, showed retarded development or were in a macerated condition were classed as late deaths, according to the terminology of Bateman & Epstein (1971) and the MARTA Committee on Terminology (1969). A runt was considered to be any foetus that weighed at least 30% less than the average weight of the male or female controls (Leuschner & Czok, 1973). Each live foetus was weighed, sexed and examined for gross external malformations under magnification, and the crown–rump length was measured. Corpora lutea were counted under magnification. Approximately half of the foetuses from each litter were fixed in alcohol, stained with Alizarin Red S (Dawson, 1926) and examined for skeletal variations. The remaining foetuses were fixed in Bouin's solution, razor-blade sectioned (Wilson, 1965) and examined for internal variations of the soft tissues. Microscopic examinations were carried out without the investigator's knowing to which treatment group the litters belonged.

Statistical analysis. Data on the numbers of corpora lutea per dam, implantations per dam, numbers of viable implants per dam, foetal body weights and crown–rump lengths were submitted to an analysis of variance followed by a least significant difference (LSD) test. The number of resorptions per litter and the average number of foetuses with one or more variations per litter were transformed by use of the Freeman–Tukey arc-sine transformation for binomial proportions (Mosteller & Youtz, 1961) followed by an analysis of variance and an LSD test. Preimplantation loss data were transformed using the Freeman–Tukey arc-sine transformation followed by an analysis of variance and an LSD test. The numbers of litters with one or more resorptions, one or more skeletal or soft-tissue variations and specific external, soft-tissue and skeletal variations were analysed by Fisher's exact one-tail test (Siegel, 1956). An analysis of variance was used to test maternal weight gain. An Armitage test for linearity of proportions (Armitage, 1973) was also used to detect trends. Differences in water consumption were analysed by a paired *t*-test.

RESULTS

FD & C Red No. 40 produced no external signs of toxicity; the animals appeared healthy and behaved normally.

In the drinking-water study, the average daily water consumption of the treated rats (36·3 ml) was significantly ($P < 0·05$) less than that of the controls (39·3 ml). Daily consumption of FD & C Red No. 40 as the 0·2% solution was 260·2 mg/kg body weight, an amount slightly greater than the highest dose given by intubation.

Despite the decreased water consumption at the 0·2% dose level, there was no corresponding effect on maternal weight gain (Table 1), nor was there any dose-related effect on maternal weight gain when the compound was administered by intubation. Maternal reproductive data also showed no significant changes in or treatment-related effects on the numbers of corpora lutea, implantations, early and late deaths and viable foetuses per litter or on the percentage of preimplantation loss. The percentage of early resorptions was increased slightly in groups given 7·5 or 15 mg/kg but this response appeared to be the result of sporadic occurrences. One litter of a female given 100 mg/kg was totally resorbed. However, the percentage of females with more than one and with more than two resorptions also showed no dose-related correlation. The mean foetal body weight did not vary with treatment (Table 2). The average crown–rump length of both males and females in the intubation study decreased slightly but not significantly with increasing dose level, but was not changed in the drinking-water study. The percentage of males and females per treatment group were approximately equal, except in the 0- and 200-mg/kg dose groups in which there were slightly more males than females.

No compound-related external variations were observed (Table 3). Of all the groups, the control animals for the drinking-water study had the greatest number of variations. However, a single litter contained foetuses with four of the variations—two with oedema, one with cranial pimple and one with exencephaly. In the drinking-water study, the number of runts was increased in the treated group, but in the intubation study runts were found in litters of dams at all of the dose levels except 30 mg/kg.

An assortment of specific soft-tissue variations occurred, but none appeared to be clearly dose-related. The incidence of foetuses with hydroureters was increased at the 200-mg/kg level (Table 3), but the affected foetuses were well distributed among the litters. A similar increase in hydroureters did not occur in the drinking-water study even though the daily dose was greater than that in the intubation study. The slight increase in the number of internal haemorrhages at the 15-mg/kg dose level (Table 3) appeared to be a random occurrence and not to

Table 1. *Maternal and reproductive data for rats given FD & C Red No. 40 during gestation*

Mode of administration and dose level	No. of pregnant females examined	Mean maternal body weight gain (g)*	Autopsy findings (mean/dam)					Resorptions (%)	Preimplantation loss (%)	Females with resorptions (%)	
			Corpora lutea*	Implantations*	Resorptions Early	Resorptions Late	Viable foetuses*			One or more	Two or more
Intubation (mg/kg/day)											
0	24	124·1 ± 4·5	13·0 ± 0·3	11·5 ± 0·5	1·1	0·04	10·3 ± 0·5	10·1	11·2	66·7	33·3
7·5	27	127·2 ± 5·6	13·4 ± 0·4	12·5 ± 0·4	1·4	0	11·0 ± 0·6	11·6	6·9	48·2	29·6
15	28	134·8 ± 3·9	13·8 ± 0·4	12·7 ± 0·2	1·6	0·04	11·1 ± 0·3	12·9	7·8	71·4	35·7
30	28	128·3 ± 5·1	13·4 ± 0·6	12·1 ± 0·5	1·2	0·04	10·9 ± 0·6	10·0	10·2	64·3	21·4
100	26	123·1 ± 4·4	13·6 ± 0·5	11·6 ± 0·5	0·7	0	11·0 ± 0·6	5·9	14·6	34·6	23·1
200	25	123·3 ± 4·1	13·0 ± 0·4	11·6 ± 0·4	1·0	0	10·6 ± 0·5	8·3	11·4	56·0	20·0
Drinking water (%)											
0	30	147·0 ± 3·6	13·4 ± 0·4	12·0 ± 0·5	0·5	0	11·5 ± 0·5	4·4	10·4	43·3	10·0
0·2	25	147·5 ± 4·1	13·6 ± 0·3	12·7 ± 0·3	0·6	0	12·0 ± 0·3	5·0	7·0	48·0	16·0

*Values are means ± SEM.

Table 2. *Foetal data for rats given FD & C Red No. 40 during gestation*

Mode of administration and dose level	Foetal body weight (g)*		Crown–rump length (cm)*		Sex distribution (% of live foetuses)	
	M	F	M	F	M	F
Intubation (mg/kg/day)						
0	3·87 ± 0·07	3·66 ± 0·05	4·0 ± 0·02	3·9 ± 0·02	55·2	44·8
7·5	3·85 ± 0·08	3·63 ± 0·07	3·9 ± 0·03	3·9 ± 0·03	49·0	51·0
15	3·83 ± 0·06	3·65 ± 0·05	3·9 ± 0·02	3·9 ± 0·02	51·0	49·0
30	3·86 ± 0·06	3·67 ± 0·05	3·9 ± 0·03	3·8 ± 0·03	51·2	48·8
100	3·86 ± 0·04	3·65 ± 0·06	3·9 ± 0·03	3·8 ± 0·03	48·4	51·6
200	3·83 ± 0·04	3·58 ± 0·05	3·9 ± 0·02	3·8 ± 0·03	56·2	43·8
Drinking-water (%)						
0	4·02 ± 0·05	3·78 ± 0·05	4·0 ± 0·01	3·9 ± 0·02	50·7	49·3
0·2	3·95 ± 0·05	3·77 ± 0·05	4·0 ± 0·02	3·9 ± 0·02	49·5	50·5

*Values are means ± SEM.

be associated with the ingestion of FD & C Red No. 40.

In the intubation study, there was no dose-related increase in the average number of variations per litter or in the average number of foetuses with one or more soft-tissue variations (Table 4). There was a slight, but not significant, increase in the percentage of foetuses with soft-tissue variations at 200 mg/kg, but this increase did not appear to be meaningful since the value was nearly the same as the control value in the drinking-water study. The average number of soft-tissue variations per litter was greater in the control animals of the drinking-water study than in those of the intubation study and the number of foetuses with variations was also greater, but the percentage of litters involved was approximately the same. The number of affected litters was increased slightly in the group intubated at the 200-mg/kg dose level and in the group consuming FD & C Red No. 40 in their drinking water.

Among the skeletal variations (Table 5), slight increases were found in the number of bipartite sternebrae at 7·5 mg/kg and in the number of malaligned sternebrae at the 7·5, 30 and 200 mg/kg dose levels, but these increases did not show a dose-response relationship. In the drinking-water study, the number of bipartite sternebrae was slightly increased at the 0·2% dose level compared with the control value, but the values were less than those for the corresponding groups (200-mg/kg and control, respectively) in the intubation study.

Several types of rib variations, including reduced thirteenth ribs, extra ribs or rib buds, wavy ribs and incompletely ossified ribs, were observed (Table 5). The number of animals with fourteenth rib buds was slightly increased at 7·5, 30 and 200 mg/kg, but the increases did not appear to be dose-related. Apparent increases in wavy ribs occurred at all dose levels in the intubation study. However, they were not statistically significant and were not dose-related. Moreover, the control value (0) in the intubation study appeared to be abnormally low. In the animals given the compound *via* the drinking-water, foetuses with wavy ribs were concentrated in only two litters.

There were no treatment-related effects on any specific skull bones (Table 5). At 100 mg/kg in the intubation study and at 0·2% in the drinking-water study, the incidence of reduced ossification of the hyoid bone was observed but the increase did not appear to be related to dose. The incidence of reduced ossification of the parietal bone was also slightly increased in the drinking-water study.

There was no compound-related effect on the average number of sternebral variations per litter, the number of foetuses with variations or the litter incidence (Table 6). The control values for animals in the drinking-water study were considerably lower than the control values in the intubation study.

Sporadic increases were seen in the average number of skeletal variations per litter and in the number of foetuses with one or more skeletal variations at the 7·5- and 100-mg/kg dose levels (Table 7) but there was no dose-related effect at 200 mg/kg. These sporadic variations are not considered significant, especially when the values are compared with the control values for the drinking-water study.

DISCUSSION

Only a small number of reproduction and teratogenicity studies have been carried out on FD & C Red No. 40. The rat reproduction study was included as part of a chronic study (Allied Chemical Corporation, unpublished data 1970, in FDA files), but was not started until the animals were quite old and fertility was low in the controls. The study showed little relationship between effect and dosage. This finding appears to agree with the results of the present study, in which neither embryolethality nor foetotoxicity was observed. There were slight increases in the incidence of various skeletal variations but increases were not dose-related. Most notable was the increased incidence of wavy ribs in rats given FD & C Red No. 40 by intubation but this increase could have been due to the absence of wavy ribs in the controls. It should be noted that in the drinking-water study there were only increases in the numbers of foetuses with wavy ribs and no increase in the number of litters affected. On the basis of this rat study, FD & C Red No. 40 was without apparent effect when administered to rats by gavage at dose levels of up to 200 mg/kg/day or given in drinking water at 260 mg/kg/day.

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Table 3. *Incidence of specific external and soft-tissue variations in foetuses of rats given FD & C Red No. 40 during gestation*

Variation	Dose level†...	Incidence* in foetuses and litters of rats administered FD & C Red No. 40...							
		By intubation						In the drinking water	
		0	7·5	15	30	100	200	0	0·2
External examination									
No. examined		248 (24)	298 (27)	310 (28)	305 (28)	285 (25)	265 (25)	345 (30)	301 (25)
Runt		1 (1)	2 (2)	2 (2)		2 (2)	1 (1)	1 (1)	4 (4)
Tail bud			1 (1)						
Oedema							1 (1)	2 (1)	
Cranial pimple								1 (1)	
Exencephaly								1 (1)	
Soft–tissue examination									
No. examined		133 (24)	158 (27)	163 (28)	156 (27)	148 (25)	138 (25)	180 (30)	161 (25)
Hydroureter: moderate		7 (7)	10 (7)	7 (6)	5 (3)	6 (5)	13 (9)	7 (5)	7 (5)
severe			1 (1)	1 (1)	2 (2)		1 (1)	1 (1)	1 (1)
Hydronephrosis: moderate		8 (6)	5 (4)	11 (7)	7 (6)	4 (4)	6 (5)	8 (4)	6 (5)
severe			2 (1)		2 (2)		2 (1)	1 (1)	1 (1)
Ectopic kidney						2 (2)	1 (1)	2 (2)	1 (1)
Pitted kidney				1 (1)			1 (1)	1 (1)	
Deformed kidney				1 (1)			1 (1)		
Haemorrhage		4 (4)	9 (5)	10 (9)	4 (3)	5 (5)	4 (3)	13 (8)	8 (7)
Hydrocephalus								2 (1)	1 (1)
Microphthalmia								1 (1)	
Oesophageal pouch			1 (1)					1 (1)	

*No. of foetuses affected and, in parentheses, no. of litters.
†The dose level is expressed as mg/kg/day in the intubation study or as a percentage in the drinking-water.

Table 4. *Incidence of soft-tissue variations in foetuses of rats given FD & C Red No. 40 during gestation*

Mode of administration and dose level	Soft-tissue variations		Foetuses with one or more soft-tissue variations			Litters with one or more foetuses with variations	
	Total	Mean/litter	No.	Mean/litter	% of total foetuses	No.	% of total litters
Intubation (mg/kg)							
0	19	0·8	15	0·6	11·3	11	45·8
7·5	28	1·0	20	0·7	12·7	13	48·2
15	31	1·1	22	0·8	13·5	12	42·9
30	20	0·7	15	0·6	9·6	10	37·0
100	17	0·7	14	0·6	9·5	10	40·0
200	29	1·2	22	0·9	15·9	14	56·0
Drinking water (%)							
0	37	1·2	28	0·9	15·6	13	43·3
0·2	25	1·0	17	0·7	10·6	14	56·0

Table 5. *Incidence of specific skeletal variations in foetuses of rats given FD & C Red No. 40 during gestation*

Variation	Dose level† ...	Incidence* in foetuses and litters of rats administered FD & C Red No. 40 ...							
		By intubation						In drinking-water	
		0	7·5	15	30	100	200	0	0·2
No. of foetuses examined		115 (24)	138 (27)	146 (28)	148 (28)	136 (25)	127 (25)	162 (30)	140 (25)
Sternebrae: RO		26 (19)	22 (14)	29 (15)	34 (20)	26 (15)	28 (16)	21 (14)	26 (14)
missing		29 (16)	32 (14)	36 (22)	29 (13)	30 (16)	29 (17)	31 (15)	25 (12)
bipartite		5 (5)	12 (8)	7 (6)	5 (5)	3 (3)	7 (6)	3 (3)	5 (3)
malaligned		2 (2)	6 (5)	2 (2)	6 (6)	3 (3)	5 (5)	2 (2)	2 (2)
Fourteenth rib		4 (4)	1 (1)	2 (2)	2 (2)	1 (1)		10 (8)	1 (1)
Fourteenth rib bud		2 (2)	9 (6)	2 (1)	6 (6)	2 (2)	6 (6)	10 (7)	5 (4)
Thirteenth rib bud			2 (1)		1 (1)	1 (1)	1 (1)	1 (1)	
Fifteen ribs				1 (1)					
Ribs: wavy			5 (4)	5 (4)	4 (4)	3 (3)	6 (4)	2 (2)	6 (2)
RO									1 (1)
Hyoid bone: RO		4 (3)	5 (3)	7 (5)	4 (2)	9 (6)	6 (3)	5 (3)	9 (6)
Interparietal bone: RO		17 (10)	23 (10)	22 (14)	17 (9)	28 (13)	21 (11)	24 (14)	24 (11)
Parietal bone: RO		5 (3)	5 (4)	5 (4)	2 (2)	5 (2)	4 (4)	2 (2)	8 (6)
Supraoccipital bone: RO			1 (1)					1 (1)	
bipartite							1 (1)		
Frontal bone: RO							1 (1)		1 (1)
Bone spur from L3 vertebra									1 (1)
Vertebral centrum: RO		1 (1)							
bipartite									1 (1)
Phalanges: RO		1 (1)					1 (1)		1 (1)
Metacarpals: RO								1 (1)	

RO = Reduced ossification

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

†The dose level is expressed as mg/kg/day in the intubation study or as a percentage in the drinking-water.

Table 6. *Incidence of sternebral variations in foetuses of rats given FD & C Red No. 40 during gestation*

Mode of administration and dose level	Sternebral variations		Foetuses with one or more sternebral variations			Litters with one or more foetuses with variations	
	Total	Mean/litter	No.	Mean/litter	% of total foetuses	No.	% of total litters
Intubation (mg/kg)							
0	62	2·6	56	2·3	48·7	21	87·5
7·5	72	2·7	63	2·3	45·6	21	77·8
15	74	2·6	62	2·2	42·5	25	89·3
30	74	2·6	68	2·4	46·0	26	92·9
100	62	2·5	56	2·2	41·2	22	88·0
200	69	2·8	58	2·3	45·7	23	92·0
Drinking water (%)							
0	57	1·9	49	1·6	30·2	22	73·3
0·2	58	2·3	52	2·1	37·1	20	80·0

Table 7. *Incidence of skeletal variations other than sternebral variations, in foetuses of rats given FD & C Red No. 40 during gestation*

Mode of administration and dose level	Skeletal variations		Foetuses with one or more skeletal variations			Litters with one or more foetuses with variations	
	Total	Mean/litter	No.	Mean/litter	% of total foetuses	No.	% of total litters
Intubation (mg/kg)							
0	34	1·4	25	1·0	21·7	17	70·8
7·5	51	1·9	37	1·4	26·8	15	55·6
15	44	1·6	29	1·0	19·9	17	60·7
30	36	1·3	29	1·0	19·6	15	53·6
100	49	2·0	34	1·4	25·0	17	68·0
200	47	1·9	28	1·1	22·0	14	56·0
Drinking water (%)							
0	56	1·9	42	1·4	25·9	21	70·0
0·2	58	2·3	31	1·2	22·1	14	56·0

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COMPARISON OF EFFECTS OF DIETARY ADMINISTRATION OF BUTYLATED HYDROXYTOLUENE OR A POLYMERIC ANTIOXIDANT ON THE HEPATIC AND INTESTINAL CYTOCHROME *P*-450 MIXED-FUNCTION-OXYGENASE SYSTEM OF RATS*

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Abstract—The antioxidant D00079 is a divinylbenzene–hydroquinone–phenols condensation polymer which is being developed for use in foods. D00079 or BHT was administered in the diet to adult male albino rats at levels of 2500 or 250 mg/kg body weight/day, respectively, for up to 60 days and the two antioxidants were compared for their effects on hepatic and intestinal mixed-function-oxygenase (MFO) systems. Throughout the study, the relative liver weights, hepatic cytochrome *P*-450 levels and MFO enzyme activities of the group fed D00079 were similar to those of the contro. group. In contrast, in the BHT-fed group there were significant increases in relative liver weights, in hepatic cytochrome *P*-450 content, and in hepatic *p*-nitroanisole-*O*-demethylase and aminopyrine-*N*-demethylase activities. The BHT group also showed consistently depressed benzo[*a*]pyrene hydroxylase activity in both hepatic and intestinal preparations. The concentration of BHT increased in hepatic homogenates from the BHT-fed group during the course of the study. In contrast, only trace amounts of BHT were found in the control and D00079 hepatic homogenates, and these low levels of BHT were presumed to have been derived from the basal diet. We conclude that the polymer antioxidant D00079 has no effect on MFO systems, while BHT produces significant liver enlargement and enzyme induction.

INTRODUCTION

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used to prevent rancidity of fats and oils and so to increase the shelf-life of many food products. Recent reviews of the safety of these antioxidants have revealed no consistent evidence of any significant short-term toxicity of BHT in experimental animals at dose levels more than 100 times the maximum amounts likely to be consumed by man (Branen, 1975; Daniel, 1976; Johnson, 1971). However, because of their extensive use, questions about the long-term toxicity of these compounds have arisen. Both BHA and BHT have been shown to be readily absorbed from the gastro-intestinal tract and are metabolized in many animal species, including man (Astill, Mills, Fassett, Roudabush & Jerhaar, 1962; Daniel, Gage, Jones & Stevens, 1967). This high degree of bioavailability leads to the continuous exposure of internal organs to these compounds and their metabolites and this has been shown to affect a number of enzyme systems. Among the most thoroughly studied is the microsomal mixed-function-oxygenase (MFO) system of the liver. A hypertrophic response in the liver with a concomitant increase in the cytochrome *P*-450-associated enzyme activity has been shown to occur in mice and rats (Botham, Conning, Hayes, Litchfield & McElligott, 1970; Creaven, Davies & Williams, 1966; Feuer, Gaunt, Golberg & Fairweather, 1965a; Feuer, Golberg & Le Pelly, 1965b; Feuer & Granda, 1970; Gaunt, Feuer, Fairweather & Gilbert, 1965a; Gaunt, Gilbert & Martin, 1965b; Gilbert & Golberg, 1965a,b & 1967; Martin & Gilbert, 1968). Although the inductive response is reversible once exposure is discontinued, the effect of the microsomal-enzyme induction on the metabolism of other potentially harmful compounds to which the animals might be exposed simultaneously is not clear. The life-long dietary consumption of these additives could prove to have previously unsuspected deleterious effects in man (Food and Drug Administration, 1977a,b).

The polymer antioxidant D00079 has been shown to be essentially nonabsorbed from the gastro-intestinal tract (Parkinson, Honohan, Enderlin, Halladay, Hale, de Keczer, Dubin, Ryerson & Read, 1978; Walson, Carter, Ryerson, Halladay & Parkinson, 1979). In order to determine whether this reduction in bioavailability limits the induction of hepatic and intestinal microsomal enzymes, the effects of feeding D00079 and BHT to rats daily for 60 days on liver size and cytochrome *P*-450-associated enzyme systems were compared.

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EXPERIMENTAL

Materials. Butylated hydroxytoluene (>95% pure) was a gift from the Ashland Chemical Co. (Columbus, OH). The D00079 antioxidant is a divinylbenzene–hydroquinone–phenols condensation polymer prepared at Dynapol (Parkinson *et al.* 1978). Gel-permeation chromatography indicated a peak molecular weight of approximately 5000 relative to polystyrene standards. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma Chemical Co., St. Louis, MO. The other chemicals and solvents used were reagent grade.

Treatment. The investigation was conducted in two parts. In study I, male S/A Simonsen albino rats (Simonsen Laboratories, Inc., Gilroy, CA), 27–32 days old and weighing 110–120 g, were given D00079 dissolved in corn oil and mixed in Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) at a final concentration of 5% by weight, in the diet (approximately 2500 mg/kg body weight/day) for 60 days. Control animals were fed a diet containing corn oil alone. Fresh diet was prepared every 2 wk. For study II, BHT was administered to similar rats at approximately 250 mg/kg body weight/day by grinding and mixing the BHT directly into the diet. The daily dose was based on the group mean body weight which was determined every 3–4 days. At these intervals, BHT consumption was calculated and new diets were prepared. Control animals were given Purina Laboratory Chow alone. Exposure was continued for 60 days.

Preparation of liver homogenates. In studies I and II the rats were killed by cervical dislocation after 0, 15, 30 or 60 days. Hepatic homogenates were prepared after perfusing the livers *in situ* with 50 ml cold 1·15% (w/v) KCl containing 100 mM-Tris-HCl buffer (pH 7·4) *via* the portal vein. The liver was removed, minced and homogenized in three volumes of Tris-HCl buffer. (Homogenization was carried out by three passes of a Teflon pestle, the total clearance of the pestle in the homogenizer being 0·04 in.) This crude homogenate was centrifuged at 4°C in a Sorvall centrifuge at 9000 g for 10 min. The top fatty layer was discarded and the remaining supernatant, referred to as the S-9 fraction, was used as the source of the cytochrome *P*-450 enzyme system.

To prepare the intestinal homogenate, the entire length of the small intestine was removed and rinsed inside and out with ice-cold 1·15% (w/v) KCl containing 0·1 M-Tris-HCl buffer (pH 7·4) and trypsin inhibitor (5 mg/g tissue). The intestine was cut into 5–10 cm lengths, the segments were placed on a cold glass plate, cut longitudinally and spread open, mucosal side up. The mucosal layer was scraped off with a microscope slide, weighed and placed in a cold homogenizer containing 10 ml Tris buffer. Intestinal tissue was pooled (usually 17–20 g collected from five rats) and homogenized as described above in three volumes of Tris buffer.

Enzyme assays. The cytochrome *P*-450-dependent enzyme activities in the liver S-9 fractions were determined for *p*-nitroanisole-*O*-demethylase (NADM; Kato & Gillette, 1965), benzo[*a*]pyrene hydroxylase (BH; Cantrell, Abreu & Busbee, 1976) and aminopyrine-*N*-demethylase (APDM; assayed by determining formaldehyde produced by the method of Nash, 1953). NADM and APDM activities were determined in mixtures containing 1·5 ml S-9 fraction (equivalent to 0·375 g liver), 0·025 ml $MgCl_2$ (7·5 μmol), 3·0 μmol NADPH and substrate (NADM: 9 μmol *p*-nitroanisole; APDM: 0·04, 0·1 or 0·2 μmol 4-aminopyrine). Incubation was carried out in cold Erlenmeyer flasks. For determination of BH activity in the hepatic S-9 fraction, incubation was carried out in cold 25-ml vials and the mixtures contained 1·0 ml S-9 fraction (equivalent to 0·25 g tissue), 0·025 mg (5·0 μmol) $MgCl_2$, 0·05 ml NADPH and 0·16 μmol benzo[*a*]pyrene.

The intestinal S-9 fractions were assayed for BH activity in incubation mixtures containing a volume of intestinal S-9 fraction equivalent to 0·25 g tissue, 0·025 ml (5·0 μmol) $MgCl_2$, 0·05 ml (1·5 μmol) NADPH and 0·08 μmol benzo[*a*]pyrene. Incubation was carried out in cold 25-ml vials.

All of the samples were incubated at 37°C under air, in a Dubnoff metabolic shaking incubator (GCA Precision Scientific, Chicago, IL) oscillating at 70 cycles/min. Before addition of the substrate, S-9 fraction samples were incubated for 2 min at 37°C.

Cytochrome *P*-450 was measured by the method of Schoene, Fleischman, Remmer & Von Oldershausen (1972). A 0·5 ml aliquot of the S-9 liver homogenate was diluted to 10·5 ml (final S-9 homogenate concentration 11·9 mg liver/ml) with 1·15% (w/v) KCl containing 0·1 M-Tris-HCl (pH 7·4) and gassed with CO for 4 min. For analysis, 1·0-ml samples from the gassed S-9 fraction were placed in matched 1-ml sample and reference cuvettes. The contents of the sample cuvette were reduced by the addition of approximately 2 mg of powdered sodium dithionite and were analysed by scanning wavelengths 550–400 nm. All analyses were carried out at 10°C. A molar extinction coefficient of $100\ cm^{-1}\ mM^{-1}$ for reduced *P*-450 in the S-9 fraction was used in all calculations (Estabrook, Peterson, Baron & Hildebrandt, 1972).

Extraction of BHT from tissue samples. A 5·0-ml aliquot of hepatic-tissue S-9 fraction was extracted twice with 5 ml heptane. The extracts were combined from the appropriate feeding intervals, dried over anhydrous Na_2SO_4, and evaporated under a stream of nitrogen. The residue was redissolved in a known volume of *n*-heptane.

Standards were obtained by the same extraction of untreated tissue samples after the addition of known quantities of BHT, and of 2-phenyl phenol (internal standard). A Finnigan 1015 (Sunnyvale, CA) gas chromatograph–mass spectrometer (in the electron impact mode, 70 eV) was used. The column (6 ft long, 2 mm ID) was packed with 3% OV-17 and 0·3% Carbowax 20M TPA on Chromosorb W and the oven temperature was 130°C. The 205 mass fragment was monitored to obtain the final quantitative values of BHT in the tissue extracts.

Calculations and statistical tests. The concentrations of the enzyme products were determined from standard plots which were obtained by assaying the various amounts of product in the presence of the respective substrate in homogenates that had no enzymatic activity. Standards for NADM and APDM were plotted as absorbance *v.* concentration of the product in each sample. For BH, standards were plotted as log relative intensity *v.* log concentration of

Table 1. *Effects of feeding* c. *2500 mg D00079/kg body weight/day on the hepatic and intestinal cytochrome* P-*450 mixed function oxygenase systems of male S/A Simonsen albino rats*

Treatment (no. of rats)	Relative liver weight (g/100 g body weight)	Activity† of hepatic NADM	APDM	BH	Hepatic cytochrome *P*-450 (nmol/g liver)	Activity† of intestinal BH
			Day 0			
Control (5)	4·73 ± 0·50	714 ± 30	738 ± 22	92 ± 11	5·30 ± 0·70	92·2
			Day 15			
Control (5)	5·07 ± 0·44	2001 ± 358	1048 ± 242	84 ± 12	5·51 ± 1·07	50·5
D00079 (5)	5·61 ± 0·61	2058 ± 204	1128 ± 295	81 ± 13	5·35 ± 1·31	42·3
			Day 30			
Control (5)	4·37 ± 0·69	1456 ± 192	730 ± 209	112 ± 17	4·49 ± 1·38	24·1
D00079 (5)	4·91 ± 0·45	1561 ± 302	880 ± 216	139 ± 15*	4·70 ± 0·55	32·1
			Day 60			
Control (5)	3·80 ± 0·16	1608 ± 440	869 ± 180	149 ± 33	4·47 ± 0·82	19·0
D00079 (5)	3·66 ± 0·30	1735 ± 435	974 ± 99	143 ± 28	3·16 ± 0·39**	16·0

NADM = *p*-Nitroanisole-*O*-demethylase APDM = Aminopyrine *N*-demethylase
BH = Benzo[*a*]pyrene hydroxylase

†Enzyme activities are expressed as follows: NADM—nmol *p*-nitrophenol/g liver/hr; APDM—nmol formaldehyde/g liver/hr; BH—nmol 3-hydroxybenzo[*a*]pyrene/g liver (or intestinal tissue)/hr.

Values are means ± 1SD for the number of rats indicated and those marked with asterisks differ significantly (Student's *t*-test) from the corresponding control values (*$P < 0.05$; **$P < 0.01$). For determinations of intestinal BH activity the tissue samples from all of the rats in each group were combined.

product in each sample. Standard plot line slopes were calculated using a Tektronix Line Fitting Program (1973). Enzyme activities were expressed as nmol/product/g tissue/hour.

Statistical tests were used to determine the significance of differences in enzyme activities, body weights, and relative liver weights between treatment and control animals. An F-test was used to check for within group variance at the 0·01 probability level. The significance of differences was assessed by the Student's *t*-test. A *P* value of $\leqslant 0.05$ was considered significant.

RESULTS

The results are summarized in Tables 1 and 2 and the changes in each measurement that occurred during the feeding periods are shown in Figs 1 and 2. Significant increases ($P < 0.05$) in relative liver weights were seen at all sampling periods for animals fed BHT. No significant increases in liver weight were observed in animals exposed to D00079 (Fig. 1).

The concentrations of cytochrome *P*-450 were similar in the liver homogenates from control rats and from rats fed D00079 except on day 60, when *P*-450

Table 2. *Effects of feeding* c. *250 mg BHT/kg body weight/day on the hepatic and intestinal cytochrome* P-*450 mixed function oxygenase systems of male S/A Simonsen albino rats*

Treatment (no. of rats)	Relative liver weight (g/100 g body weight)	Activity† of hepatic NADM	APDM	BH	Hepatic cytochrome *P*-450 (nmol/g liver)	Activity† of intestinal BH
			Day 0			
Control (5)	4·19 ± 0·49	1133 ± 308	1324 ± 494	208 ± 139	5·10 ± 1·40	41·1
			Day 15			
Control (5)	4·42 ± 0·18	2268 ± 442	993 ± 259	425 ± 79	4·92 ± 1·61	11·6
BHT (5)	6·35 ± 0·19****	3019 ± 403*	1366 ± 265	208 ± 54****	5·34 ± 1·67	3·4
			Day 30			
Control (5)	4·29 ± 0·30	1885 ± 513	1610 ± 359	104 ± 16	5·22 ± 1·15	9·7
BHT (5)	5·72 ± 0·78***	3275 ± 625***	1689 ± 329	80 ± 24	5·96 ± 0·56	5·6
			Day 60			
Control (5)	3·55 ± 0·26	1500 ± 85	707 ± 86	197 ± 32	3·12 ± 0·77	29·3
BHT (4)	5·40 ± 0·83*	2564 ± 502*	1246 ± 282*	186 ± 34	5·00 ± 1·11**	11·2

BHT = Butylated hydroxytoluene NADM = *p*-Nitroanisole-*O*-demethylase
APDM = Aminopyrine *N*-demethylase BH = Benzo[*a*]pyrene hydroxylase

†Enzyme activities are expressed as follows: NADM—nmol *p*-nitrophenol/g liver/hr; APDM—nmol formaldehyde/g liver/hr; BH—nmol 3-hydroxybenzo[*a*]pyrene/g liver (or intestinal tissue)/hr.

Values are means ± 1SD for the number of rats indicated and those marked with asterisks differ significantly (Student's *t*-test) from the corresponding control values (*$P < 0.05$; **$P < 0.02$; ***$P < 0.01$; ****$P < 0.001$). For determination of intestinal BH activity the tissue samples from all the rats in each group were combined.

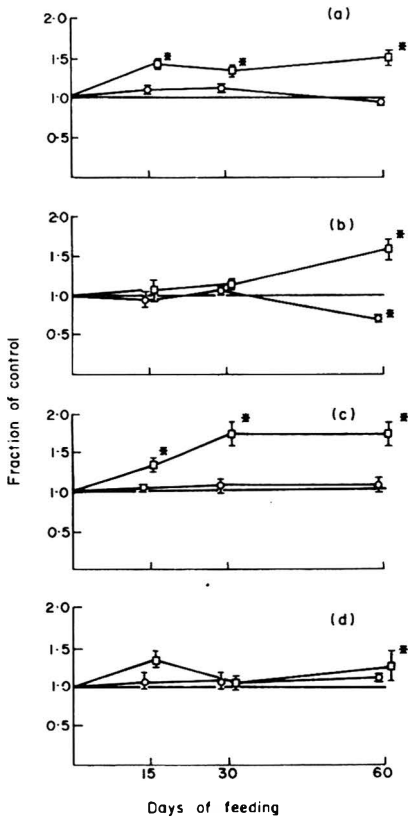

Fig. 1. Effects of feeding BHT (□) or D00079 (○) on (a) relative liver weights, (b) hepatic levels of cytochrome *P*-450, (c) the activity of *p*-nitroanisole-*O*-demethylase and (d) the activity of aminopyrine-*N*-demethylase. Values are means ±SEM for groups of five rats (four rats in the BHT-treated group after 60 days) and those that differ significantly (Student's *t*-test) from the corresponding control values at any level of *P* that is ⩽0·05 are marked with an asterisk.
Note: Data points are offset ±0·75 days for clarity.

levels in the treated rats were slightly lower ($P < 0{\cdot}02$) than in the controls (Fig. 1). In contrast, *P*-450 concentrations in the liver homogenates from BHT-fed rats showed a trend of induction throughout the feeding period and this became statistically significant ($P < 0{\cdot}02$) at day 60.

Significant increases in NADM activity were measured on days 15, 30 and 60 of BHT exposure, while NADM activity in liver homogenates from animals that were fed D00079 did not change significantly compared to controls during the test period. A significant increase (76%) in APDM was also seen in BHT-fed animals on day 60, whereas APDM-activity in D00079-fed animals remained comparable to that of the controls throughout the study (Fig. 1).

Unexpectedly, hepatic BH activity in BHT-fed rats was greatly depressed (−49%) on day 15, but was not significantly different from that of the controls on days 30 and 60 (Fig. 2). In contrast, BH activity in D00079-fed rats was similar to that of the controls except for a significant increase on day 30. There were also indications that BHT given in the diet lowered intestinal BH activity at all sampling periods, but feeding D00079 had no significant effect on intestinal BH levels.

There was a general increase with treatment time in the BHT concentration in the livers of BHT-treated rats (Fig. 2). At 60 days in both D00079-treated and control hepatic samples, BHT concentrations were found to be near the lower limits of detection, being 0·04 and 0·07 ng/g hepatic tissue, respectively. These trace quantities of BHT may have originated from the Purina Laboratory Chow or the corn oil that was added to the diets.

DISCUSSION

The polymer antioxidant (D00079) fed at a level of 5% by weight in the diet caused no liver enlargement or consistent induction of hepatic or intestinal MFO enzymes after 60 days of exposure. In contrast, BHT elevated cytochrome *P*-450 levels, caused significant liver enlargement and significantly increased NADM activity at all sampling intervals. Induction of APDM activity also occurred. BH activity was lower in the hepatic and intestinal tissues of BHT-treated animals, suggesting that residual BHT or its metabolites present in the homogenates of these tissues might have been inhibiting the *in vitro* metabolism of benzo[*a*]pyrene. A similar inhibition of BH activity *in vitro* by BHA and BHT has been reported by Yang, Strickhart & Woo (1974).

Our results are similar to those of Gilbert & Golberg (1965a,b) and confirm their reports of the effects of BHT on relative liver weight, and on NADM and APDM activities. Some differences in the magnitude of the effects may be the result of using different strains of rats, a different dose level of BHT, different vehicles of administration of the antioxidant, and different tissue preparations as the sources of enzyme activity. Gilbert & Golberg (1965b) used male and female Carworth SPF rats and female Porton SPF rats that were given BHT dissolved in arachis oil daily by stomach tube at a dose level of 500 mg/kg body weight. We used male Simonsen rats and mixed BHT into the diet to obtain a dose level of about 250 mg/kg/day. These workers used whole tissue homogenates; we used liver and gut S-9 fractions.

In BHT-fed rats the residual levels of BHT in the hepatic S-9 homogenates showed a time-dependent increase during treatment. The trace levels of BHT that were found in the hepatic tissue of the control and D00079-treated rats indicated a small but measurable exposure of the animals to BHT. This finding is not entirely unexpected considering the extensive use of BHT as an antioxidant in fats and oils that might be used in commercially prepared diets.

The activities of NADM, BH and APDM and the hepatic cytochrome *P*-450 levels were similar in the D00079-treated and the control groups. Intestinal BH activity also remained similar to control values throughout the study. The statistically significant differences in some values that occurred at certain times in some of the assays did not seem to follow any consistent trend and are of questionable significance. It should be noted that since D00079 is not significantly absorbed from the intestinal tract (Parkinson *et al.* 1978; Walson *et al.* 1979) the concentration of

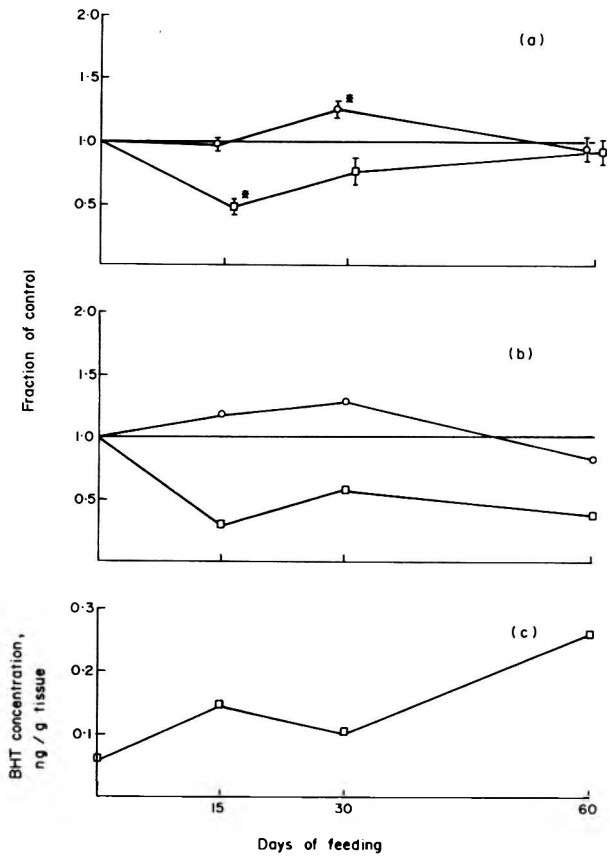

Fig. 2. Effects of feeding BHT (□) or D00079 (○) on (a) hepatic and (b) intestinal benzo[*a*]pyrene hydroxylase (BH) activity and (c) on hepatic levels of BHT. The values for hepatic BH activity are means ±SEM for groups of five rats (four rats in the BHT-treated group after 60 days) and those that differ significantly (Student's *t*-test) from the corresponding control values at any level of *P* that is ⩽0·05 are marked with an asterisk. Values for intestinal BH activity and hepatic BHT levels are for pooled samples of tissue from the number of rats indicated above.
Note: Data points are offset ±0·75 days for clarity.

this polymeric antioxidant in the gut is much higher than the 5% by weight that is present in the diet. This is due to the usual digestive and absorptive processes which remove small molecules from the lumen, leaving undigested and high-molecular-weight materials behind. The quantity of D00079 in the caecum of rats fed D00079 at 5% by weight in the diet in another study was 53 ± 8% of the dry caecal contents (Brown, Brown, Hyde & Bakner, 1978). Thus, even at extremely high concentrations, D00079 has no effect on the enzymic measurements made.

In conclusion, we have found no significant effects of D00079 on liver weight or on hepatic or intestinal mixed-function-oxygenase systems, while BHT increased liver weight and caused enzyme induction and *in vitro* inhibition of enzyme activities after *in vivo* treatment.

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SYNCARCINOGENIC ACTION OF SACCHARIN OR SODIUM CYCLAMATE IN THE INDUCTION OF BLADDER TUMOURS IN MNU-PRETREATED RATS

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Abstract—The potential of saccharin, sodium cyclamate or calcium carbonate to promote bladder cancer was investigated. Single doses of 2 mg *N*-methyl-*N*-nitrosourea (MNU) were instilled into the bladders of female rats. One group was maintained thereafter on a control diet. On the same day the three other groups of MNU-treated rats were started on a diet supplemented with 2% saccharin, 2% sodium cyclamate or 3% calcium carbonate and maintained on this diet for the rest of their lives (the doses of saccharin and sodium cyclamate were increased to 4% after 10 wk). There was no significant difference between any of the groups in average survival time, body-weight gain or food intake. All the MNU-treated groups developed urinary-tract neoplasms but there were no significant differences in tumour incidence between the MNU-treated group and the combined-treatment groups. However there were slight increases in the incidences of tumours in all the combined treatment groups, compared with that in the group given MNU alone. No significant difference in the latency periods of the tumours was found between any of the MNU-treated groups.

INTRODUCTION

Much controversy has been generated by the findings that the ingestion of some widely-used artificial sweeteners such as saccharin and cyclamate can lead to cancer of the urinary bladder in rats under specific conditions (Friedman, Richardson, Richardson, Lethco, Wallace & Sauro, 1972; Hicks, Wakefield & Chowaniec, 1975; Kroes, Peters, Berkvens, Verschuuren, De Vries & van Esch, 1977; Munro, Moodie & Grice, 1974; Oser, Carson, Cox, Vogin & Sternberg, 1975; Price, Biava, Oser, Vogin, Steinfield & Ley, 1970). Saccharin administered in the diet at high levels (5% or more) over two generations gave rise to a low incidence of cancer of the urinary bladder in the F_1 generation male rats (D. L. Arnold, C. A. Moodie, H. C. Grice, S. M. Charbonneau & I. C. Munro, unpublished report, 1977; Taylor & Friedman, 1974). However, mice, hamsters and some strains of rats fed these high levels for a single generation did not develop such tumours (Althoff, Cardesa, Pour & Shubik, 1975: Brantom, Gaunt & Grasso, 1973; Miyaji, 1973; Schmähl, 1973). It has also been found that saccharin appears to be metabolically inert in the rat and is excreted unchanged (Fitzhugh, Nelson & Frawley, 1951; Richards, Taylor, O'Brien & Duescher, 1951; Taylor, Richards & Wiegand, 1968). In addition to the studies undertaken with saccharin alone, there are reports which suggest that the effect of saccharin on bladder tumorigenesis may be a secondary one, analogous to the promoting effects reported in skin carcinogenesis (Baird, Sedgwick & Boutwell, 1971; Boutwell, 1974). However, contrary findings have also been reported in similar experiments in which saccharin was not found to enhance carcinogenic effects (Roe, Levy & Carter, 1970; Schmähl & Krüger, 1972).

When the locally active carcinogen nitrosomethylurea (MNU) was instilled into the urinary bladder of female rats and this was followed by prolonged administration of either cyclamate or saccharin in the diet, tumours were reported that did not occur after the intravesicular administration of MNU alone (Hicks *et al.* 1975). In another study, the orally active bladder carcinogen *N*-(4-(5-nitro-2-furyl)-2-thiazolyl)-formamide (FANFT) was given at a relatively low dosage followed by either high levels of saccharin or tryptophan (Cohen, Arai & Friedell, 1978). More urinary bladder cancers were seen after the additional treatment than after treatment with FANFT alone. A two-stage mechanism for urinary bladder carcinogenesis is an attractive concept, but the nature of promotion is still unknown. Studies performed so far (Hicks, Chowaniec & Wakefield, 1978) seem to indicate that this effect may be relatively non-specific, and the present investigations were undertaken to obtain

additional information and to extend these observations to include the effects of calcium carbonate on carcinogenesis.

EXPERIMENTAL

Chemicals. *N*-methyl-*N*-nitrosourea (MNU) was supplied by the Deutsches Krebsforschungszentrum, Heidelberg (purity 99·6%), sodium saccharin came from Sherwin Williams Company (Chemical Toledo Laboratories, OH, USA. Lot S1469, 1022, 3022), sodium cyclamate from Abbott Laboratories (North Chicago, USA, List No. 5683-04-PMED), and calcium carbonate was supplied by Merck, Darmstadt. Commercially pelleted animal diets (Altromin, Lage/Lippe) incorporating saccharin, cyclamate and calcium carbonate were used.

Procedure. Female 10-wk-old Wistar/AF-Han rats having an initial average body weight of 195 g were kept under standard laboratory conditions (Makrolon cages, Type III, room temperature 22 ± 2°C; relative humidity 55 ± 5%; air change 20 times/hr). A freshly prepared solution (< 15 min storage) of MNU (2 mg) in 0·5 ml distilled water was instilled into the urinary bladder (catheter: 0·66 mm diam.). The dietary treatment was started on the same day. Fifty rats/group were given saccharin (2%) or sodium cyclamate (2%) in their diet (after 10 wk, the level was increased to 4%); calcium carbonate was administered at 3% in the diet of a third group. Normal diet (Altromin Standard Diät tpf 1320, Lage/Lippe) was given to untreated controls (100 rats), to a group instilled (by bladder catheter) with water only (50 rats) and to a further group treated with MNU only (50 rats). The experiment was ended after 2 yr. On spontaneous death, complete autopsies were performed; moribund animals were killed and organs were fixed in 10% buffered formalin. Twelve graded sections were cut from each kidney, ureter and urinary bladder, embedded in paraplast blocks and stained with haematoxylin and eosin.

Statistics. Survival times were compared by Student's *t* test and tumour frequencies were compared by the chi-square test.

RESULTS

There was no significant difference ($P > 0·05$) in average survival times between the groups receiving a single intravesicular instillation of MNU alone or followed by $CaCO_3$, saccharin or cyclamate administration in the diet for life. Average body-weight gain did not differ significantly between the groups, neither did food intake or drinking water consumption (Table 1). The mean daily intake of artificial sweeteners was 2·3 g/kg body weight.

Animals that survived more than 10 wk after administration of MNU exhibited a wide range of pathological changes in the urinary tract. Necroses, haemorrhages and calcium deposits were seen in the collecting ducts of the renal papillae; dysplasia and focal hyperplasia of the papillary and renal pelvic urothelium were frequent. Hyperplasia showed more than four cell layers and dysplastic lesions demonstrated additional changes in cellular morphology.

Neoplasms with varying degrees of differentiation were seen in the urinary bladder, ureter and renal pelvis (Table 2); they were of transitional cell origin and included papillomas and carcinomas (Table 3). Malignant growth and invasion occurred simultaneously. Less differentiated transitional cell carcinomas and areas of squamous differentiation were found. In the ureter, most of the tumours (papillomas and papillary carcinomas) were found near the renal pelvis or the urinary bladder. The tumours of the urinary bladder often originated in the trigonal half of the lumen and led to extreme dilation of the ureters and renal pelvis and to hydronephrotic changes in the kidney.

All MNU-treated groups developed urinary tract neoplasms. Table 2 shows the distribution and incidence of tumours in the renal pelvis, ureters and uri-

Table 1. *Body weight, food and additive intake*

Treatment	Week no.	No. of animals	Body weight (g)*	Food intake (g/kg body weight/day)	Additive intake (g/kg body weight)
Untreated controls	0	100	204·8 ± 11·5	68·02	—
	52	99	301·8 ± 31·9	52·88	—
	104	59	323·0 ± 48·9	51·98	—
Water-instilled controls	0	50	203·4 ± 19·4	66·85	—
	52	47	298·4 ± 36·3	50·64	—
	104	28	322·5 ± 51·2	50·42	—
MNU only	0	50	194·2 ± 13·6	69·67	—
	52	38	281·1 ± 28·9	58·38	—
	104	13	319·2 ± 42·9	51·50	—
MNU + calcium carbonate	0	50	194·6 ± 16·5	70·63	2·12
	52	43	292·8 ± 32·9	55·70	1·67
	104	15	315·7 ± 37·6	58·82	1·76
MNU + saccharin	0	50	192·1 ± 17·8	69·68	1·39
	52	43	270·4 ± 22·8	60·98	2·44
	104	14	292·1 ± 45·6	63·68	2·55
MNU + sodium cyclamate	0	50	193·1 ± 19·8	70·28	1·41
	52	43	257·0 ± 22·6	63·50	2·54
	104	14	291·1 ± 31·6	59·70	2·39

*Values are means ± SD.

Table 2. *Tumour distribution in the urinary tracts of rats given various additive treatments*

Treatment	No. of rats	Survival (wk)	No. of animals with tumours	No. of rats with urinary tract neoplasms in only the:						
				Renal pelvis	Ureter	Urinary bladder	Renal pelvis + ureter	Renal pelvis + bladder	Ureter + bladder	Renal pelvis + ureter + bladder
Untreated controls	100	98 ± 15	1	1	—	—	—	—	—	—
Water-instilled controls	50	93 ± 20	1	—	—	1	—	—	—	—
MNU only	49	76 ± 29	28	4	2	8	2	4	6	2
MNU + calcium	49	86 ± 23	32	11	—	10	2	5	3	1
MNU + saccharin	50	78 ± 25	35	11	2	7	3	6	3	3
MNU + sodium cyclamate	50	81 ± 27	35	11	1	14	—	4	3	—

nary bladder. Treatment with MNU alone, followed by feeding with calcium carbonate, saccharin or sodium cyclamate resulted in slightly higher incidences of urinary tract tumours than was seen with MNU alone but these differences were not statistically significant (chi-square test). In the groups fed calcium carbonate or artificial sweeteners, tumours of the renal pelvis were found with equal frequency. The number of rats with urinary bladder tumours was approximately the same in all the MNU-treated groups.

The mean tumour latency periods are shown in Table 4. No statistically significant differences in tumour latency periods were found between the MNU-treated rats and the rats given combined treatments (chi-square test: $P > 0{\cdot}05$). In the group treated with saccharin, the first tumours were found 14 wk after the start of treatment in the urinary bladder and ureter. Two weeks later in the group instilled with MNU only, the first neoplasm was detected. In those groups fed calcium carbonate or cyclamate, the first tumours were found after 26 and 30 wk respectively. However, the shortest mean tumour latency period for the ureter was in the calcium-carbonate group (53 ± 29 wk) and for the urinary bladder tumours in the groups treated with MNU only (69 ± 33 wk).

In the control groups, one papilloma of the renal pelvis (untreated control) and one papilloma of the urinary bladder (H_2O control) were observed (Table 3).

DISCUSSION

In our experiments, the dose of MNU which previously induced few, if any, bladder neoplasms (Hicks & Wakefield, 1972) resulted in a high incidence of urinary tract neoplasms in female rats. This incidence was not significantly altered by subsequent treatment with saccharin, sodium cyclamate or calcium carbonate. In addition to the tumours of the urinary bladder (Hicks, Wakefield & Chowaniec, 1975), we found that intravesicular instillation of MNU also induced neoplasms and non-neoplastic lesions in the renal pelvis and ureter. However, the unexpectedly high response to the initiating dose of MNU makes it impossible to evaluate tumour promotion by saccharin or cyclamate in the urinary tract. Calcium carbonate administration produced findings similar to those with the sweeteners. In our opinion, if there is an effect, then it is non-specific.

Before assessing the risk of the possible promoting activity of saccharin, comparison with results from other experimental models is essential. Positive data have been obtained in studies where both the initiator and promoter were fed (Cohen *et al.* 1978). This study has the additional advantage that the reactions of both sexes were studied. These investigations showed that, as well as saccharin, tryptophan also had a promoting effect which again emphasizes the need to determine the degree of specificity, the nature and the mechanisms of this biological phenomenon. Data obtained to date are far from conclusive, and further studies using both saccharin and other orally administered compounds are necessary to provide a clearer understanding of the respective roles of promotion and initiation in urinary bladder carcinogenesis.

Table 3. *Type of urinary tract neoplasms in rats given various additive treatments*

Treatment	No. of rats with neoplasms of the:					
	Renal pelvis		Ureter		Urinary bladder	
	P	C	P	C	P	C
Untreated controls	1	—	—	—	—	—
Water-instilled controls	—	—	—	—	1	—
MNU only	4	8	7	4	9	10
MNU + $CaCO_3$	4	15	3	3	9	10
MNU + saccharin	4	17	9	2	12	7
MNU + cyclamate	1	14	4	—	11	10

P = Papilloma C = Carcinoma

Table 4. *Tumour latency periods* in rats given various additive treatments*

Treatment	No. of affected rats	Tumour latency period (wk)	
		Mean ± SD	Range
Renal pelvis tumours			
Untreated controls	1	107	—
Water-instilled controls	—	—	—
MNU only	12	87 ± 25	22–106
MNU + $CaCO_3$	19	85 ± 18	47–107
MNU + saccharin	21	78 ± 20	47–106
MNU + cyclamate	15	91 ± 16	58–107
Ureter tumours			
Untreated controls	0	—	—
Water-instilled controls	0	—	—
MNU only	11	56 ± 32	16–106
MNU + $CaCO_3$	6	53 ± 29	26–106
MNU + saccharin	11	56 ± 22	14–78
MNU + cyclamate	4	63 ± 33	30–96
Urinary bladder tumours			
Untreated controls	0	—	—
Water-instilled controls	1	50	—
MNU only	19	69 ± 33	16–106
MNU + $CaCO_3$	19	82 ± 27	26–107
MNU + saccharin	19	77 ± 27	14–107
MNU + cyclamate	21	84 ± 23	37–107

*The tumour latency period is the duration of treatment of the tumour-bearing animals.

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MUTAGENIC ACTIVITY OF PYRAZINE DERIVATIVES: A COMPARATIVE STUDY WITH *SALMONELLA TYPHIMURIUM*, *SACCHAROMYCES CEREVISIAE* AND CHINESE HAMSTER OVARY CELLS

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Abstract—Three short-term assays were used to examine pyrazine and four of its alkyl derivatives (2-methylpyrazine, 2-ethylpyrazine, 2,5-dimethylpyrazine and 2,6-dimethylpyrazine) for the presence of mutagenic activity. Exposure of *Salmonella typhimurium* cultures to these compounds in an agar overlay did not result in the induction of revertants to histidine prototrophy, even when toxic doses were used. However, cultures of stationary phase *Saccharomyces cerevisiae* strain D5 showed an increase in aberrant colonies (but not mitotic recombinants) after exposure to each of the pyrazine compounds. Pyrazine and its derivatives all induced a significant (7- to 57-fold) increase in the frequency of chromosome aberrations (breaks and exchanges) in Chinese hamster ovary cells. These results indicate the need to use several assays and organisms when testing chemicals for the presence of mutagenic activity.

INTRODUCTION

Amine and carbonyl compounds are precursors in uncooked foods of many substances responsible for pleasant tastes and aromas (Maga & Sizer, 1973). Among the compounds produced by condensation of hexoses and amino acids at elevated temperatures are the simple alkyl- and alkoxypyrazines. These compounds are thought to be responsible for roasted and smoky flavours (Koehler, Mason & Newell, 1969). Since other nitrogen heterocyclics such as 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole have been shown to have mutagenic activity (Sugimura, Kawachi, Nagao, Mahagi, Seino, Shudo, Okamoto, Kosuge, Tsuji, Itai, Iitaki & Wakabayashi, 1977), and because of the wide distribution of pyrazine derivatives in food products, we have submitted pyrazine and four of its alkyl derivatives to three mutagenic assays. The effects of pyrazine and its derivatives on the frequency of revertants of *Salmonella typhimurium*, the mitotic recombination of *Saccharomyces cerevisiae* and the chromosomal aberrations of Chinese hamster ovary (CHO) cells were tested. These tests, with their different endpoints and subjects, should suffice to declare whether compounds are mutagenic or not.

EXPERIMENTAL

Chemicals. Pyrazine, 2-methylpyrazine, 2-ethylpyrazine, 2,5-dimethylpyrazine and 2,6-dimethylpyrazine were obtained from Aldrich Chemical Co., Milwaukee, WI and used without further purification. Their degree of contamination with other compounds was determined by gas–liquid chromatography, and each sample was more than 98% pure.

Chromosome aberration test. Chinese hamster ovary (CHO) cells were grown in minimum essential medium (MEM) supplemented with 15% foetal calf serum, antibiotics (streptomycin sulphate 29·6 μg/ml, kanamycin 100 μg/ml and fungizone 2·5 μg/ml) and 7·5% sodium bicarbonate (10 ml per 800 ml medium). For each chromosome aberration experiment, approximately 140,000 CHO cells were seeded on 22 mm² coverslips in 3·5 cm plastic dishes (Falcon, Cockeysville, MD, USA). Experiments were begun when cells were 60–80% confluent. For estimating the frequency of chromosome aberrations, cells were sampled 20 hr after the completion of treatment with chemicals. Four hours prior to sampling, 0·1 ml of a colchicine solution (0·01% in 2·5% MEM) was added. Cells were then treated with 1% sodium citrate solution for 10 min followed immediately by fixation in ethanol:acetic acid (3:1) for 10 min. Air-dried slides were stained with 2% orcein in 50% acetic acid:water, dehydrated and mounted. For each sample, about 100 to 200 metaphase plates were analysed for chromosome breaks and exchanges.

Saccharomyces cerevisiae mutagenicity test. Test solutions were assayed for their ability to induce mitotic cross-over in cultures of *S. cerevisiae* strain D5. The procedure used was a modification of the technique described by Zimmermann (1973). Cultures were grown for 4 days at 30°C on synthetic complete agar plates containing 5 mg adenine/litre. White colonies were isolated from these plates and suspended in a sodium phosphate buffer (pH 7·0) at a cell concen-

Table 1. *Chromosome aberrations of CHO cells exposed for 3 hr to various pyrazine derivatives with and without microsomal activation (S9 mixture)*

Chemical	Activation system*	Dilution (mg/ml)...	Percentage of metaphase plates with chromosome aberrations and in parenthesis average no. of exchanges/metaphase plate					
			40	25	20	10	5	2·5
Pyrazine	–		T	31·7 (2·03)	4·1 (0·02)	1·0 (0·00)	0·6 (0·00)	0·5 (0·00)
	+S9		T	34·4 (2·23)	6·3 (0·03)	1·4 (0·00)	0·7 (0·00)	0·6 (0·00)
2-Methylpyrazine	–		28·0 (2·51)	6·6 (0·03)	1·4 (0·00)	0·8 (0·00)	0·0 (0·00)	0·5 (0·00)
	+S9		30·7 (2·42)	8·9 (0·05)	1·8 (0·00)	1·0 (0·00)	0·5 (0·00)	0·5 (0·00)
2-Ethylpyrazine	–		T	T	T	T	5·4 (0·10)	0·6 (0·01)
	+S9		T	T	T	T	4·9 (0·08)	0·5 (0·00)
2,5-Dimethylpyrazine	–		44·9 (3·91)	4·1 (0·05)	1·1 (0·00)	0·5 (0·00)	0·6 (0·00)	0·4 (0·00)
	+S9		41·3 (3·22)	3·8 (0·03)	0·9 (0·00)	0·4 (0·00)	0·5 (0·00)	0·6 (0·00)
2,6-Dimethylpyrazine	–		T	T	T	28·6 (1·68)	4·7 (0·02)	1·0 (0·00)
	+S9		T	T	T	21·1 (0·89)	4·7 (0·01)	0·9 (0·00)

T = Toxic (chromosome aberrations could not be analysed because of mitotic inhibition and general toxic effects)

*The activating capacity of the S9 mixture was tested on the precarcinogen aflatoxin B_1 (10^{-5} M). The percentage metaphase plates with chromosome aberrations following aflatoxin B_1 treatment without S9 was 1·1% as compared to 69·8% with S9. The S9 mixture alone did not elevate the frequency of chromosome aberrations above the background level.

Table 2. *Effect of pyrazine and pyrazine derivatives on the mutation frequency of* S. typhimurium

Treatment	Concentration (mg/plate)	No. of revertant colonies/plate*					
		TA100		TA1537		TA98	
		+S9	−S9	+S9	−S9	+S9	−S9
Buffer	–	148	153	19	18	76	72
Pyrazine	100	112	81	23	10	67	63
	50	128	117	21	9	63	56
	25	101	108	17	7	65	61
	12·5	131	112	27	9	67	62
	6·3	125	154	18	8	64	62
2-Methylpyrazine	100	58	56	5	2	55	58
	50	86	92	17	3	79	62
	25	146	96	14	6	79	86
	12·5	139	127	18	9	82	79
	6·3	125	152	16	10	85	82
2-Ethylpyrazine	100	24	12	T	T	5	3
	50	91	115	11	4	66	40
	25	126	114	16	8	70	66
	12·5	117	165	12	12	63	68
	6·3	119	155	17	16	75	64
2,5-Dimethylpyrazine	200	78	84	7	1	35	58
	100	129	114	8	8	59	54
	50	142	145	15	8	56	71
	25	160	170	17	7	59	56
	12·5	166	136	12	10	59	67
2,6-Dimethylpyrazine	100	82	70	9	14	49	58
	50	108	118	20	12	69	73
	25	112	100	16	11	73	75
	12·5	125	130	14	7	63	72
	6·3	163	164	21	8	71	71
2-Nitrofluorene	5×10^{-3}	—	—	—	—	—	1472
Benzo[*a*]pyrene	10^{-2}	301	113	—	—	—	—
9-Aminoacridine	10^{-1}	—	—	—	991	—	—

T = Toxic

*Results are the average of three replicate plates. Each treatment was performed a minimum of twice. The S9 mix consisted of 33 μl liver supernatant from Aroclor 1254-treated rats/ml S9 mix.

tration of 10^8 cells/ml. Aliquots (0·1 ml) of this cell suspension were placed into sterile test tubes. Dilutions of test compounds were prepared and 0·9 ml aliquots were immediately added to these test tubes. Samples were incubated in a shaker water-bath at 30°C for 4 hr. Each sample was then diluted with sterile distilled water to a cell concentration of 400 cells/ml and 0·5 ml of this dilution was plated onto synthetic complete agar plates (Zimmermann, 1973). Agar plates were incubated for 7 days at 30°C prior to visual scoring for colour development of colonies. The presence of a red or pink pigmentation of colonies indicated the occurrence of one of several types of gene mutation, including gene conversion, point mutations, chromosome deletions and aneuploidy. When both pink and red colour pigments were present in the same colony, the colony was scored as a mitotic recombinant (Zimmermann, 1973). Total colonies per plate were scored after 3 days incubation by using an Artek Automatic Colony Counter (Fisher Scientific Co., Ltd., Vancouver, BC).

Salmonella typhimurium *mutagenicity test.* The histidine-requiring strains TA98, TA1537 and TA100 of *Salmonella typhimurium* were used as indicator organisms for mutagenic activity. Assays for *his*$^+$ reversion frequency in the presence and absence of microsomal activation were performed by exposing bacteria at 37°C to chemicals in an agar overlay as described by Ames, McCann & Yamasaki (1975).

Microsomal activation mix for chromosome studies. Microsomal liver preparations were obtained from Swiss male rats which had been pretreated with Aroclor 1254 (Ames *et al.* 1975). The S9 activation mix consisted of liver supernatant (0·3 ml/ml mix), 0·4 M-$MgCl_2$ (0·02 ml/ml mix), 1·65 M-KCl (0·02 ml/ml mix), 6-glucosophosphate (1·3 mg/ml mix), NADP (2·55 mg/ml mix) and phosphate-buffered saline, pH 7·4 (0·62 ml/ml mix). This solution was freshly prepared and 0·5-ml aliquots added to each petri dish prior to addition of test chemical (0·5 ml).

RESULTS

The chromosome breaking capacity of pyrazine and four alkyl derivatives is shown in Table 1. All of the test compounds induced chromosome breaks and exchanges. However, the range of pyrazine concentrations which produced a positive result was relatively narrow. The standard S9 activation mixture did not alter significantly the chromosome breaking capacity of the pyrazines.

Incubation of *S. typhimurium* cultures with pyrazine or one of the derivatives in an agar overlay did not increase the reversion frequency to histidine prototrophy of any of the tester strains (Table 2). A range of doses of each sample was used (toxic and non-toxic). Furthermore, the presence of a liver microsomal activation mix (+S9 mix) in the agar overlay did not result in the induction of any histidine revertants.

S. cerevisiae cultures which were incubated in solutions of the pyrazine samples showed an increase in the proportion of surviving cells which developed into aberrant (mutant) colonies. However, mitotic recombinants (identified by the presence of both red and pink segments in a single colony) were absent (Table 3).

DISCUSSION

Pyrazine and its four examined derivatives induced chromosome breaks and exchanges in CHO cells fol-

Table 3. *Effect of pyrazine and pyrazine derivatives on the mutation frequency of* S. cerevisiae *strain D5*

Treatment	Concentration (mg/ml)	Percentage survivors†	Percentage crossovers among survivors†	Total percentage aberrations among survivors†
Buffer	—	100 (11,940)	<0·0084 (0)	0·067 (8)
Pyrazine	60	37 (414)	<0·242 (0)	0·483* (2)
	30	112 (1243)	<0·080 (0)	0·080 (0)
	15	103 (1144)	<0·087 (0)	0·087 (1)
	7·5	99 (1098)	<0·091 (0)	0·091 (0)
2-Methylpyrazine	67·5	4 (39)	<2·56 (0)	2·56* (1)
	33·8	79 (880)	<0·113 (0)	0·568* (5)
	16·9	85 (946)	<0·106 (0)	0·211* (2)
	8·5	101 (1126)	<0·089 (0)	0·267* (3)
2-Ethylpyrazine	67·5	Toxic	—	—
	33·8	20 (221)	<0·452 (0)	0·452* (1)
	16·9	91 (1005)	<0·100 (0)	0·299* (3)
	8·5	102 (1131)	<0·088 (0)	0·177* (2)
2,5-Dimethylpyrazine	135	42 (5246)	<0·0 9 (0)	0·304* (16)
	67·5	94 (1055)	<0·095 (0)	0·095 (1)
	33·8	98 (1084)	<0·092 (0)	0·185* (2)
	16·9	92 (1026)	<0·097 (0)	0·097 (1)
2,6-Dimethylpyrazine	33·8	68 (1412)	0·071* (1)	0·283* (4)
	15	112 (1241)	<0·081 (0)	0·161* (2)
	7·5	106 (1175)	<0·085 (0)	0·255* (3)
	3·3	115 (1272)	<0·079 (0)	0·236* (3)
Ethyl methane sulphonate	9	73 (1098)	1·73* (19)	6·47* (71)

†Values in parentheses are actual colony numbers.
‡Values are pooled from several experiments.
Values marked with asterisks are greater than twice the spontaneous frequency.

lowing a 3-hr exposure with or without activation with S9 preparation. The extent of the chromosome aberrations can be best judged when compared with the action of other natural components. For example, the frequency of metaphase plates with at least one chromosome aberration was about 23% following treatment with 10^{-3} M-cysteine plus 10^{-4} M-CuII, 8% with 10^{-3} M-glutathione plus 10^{-4} M-CuII (Stich, Wei & Lam, 1978), 24% with 10^{-2} M-ascorbate, 22% with 10^{-3} M-ascorbate plus 10^{-4} CuII (Stich, Wei & Whiting, 1979), 47% with caramel powder (10 mg/ml) and between 20 and 66% following application of five different caramelized sugars (Stich, Stich, Rosin & Powrie, 1980). Moreover, the chromosome damaging capacity of pyrazine and its derivatives is comparable to that of a 10^{-5} M solution of the well-known mutagens/carcinogens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and aflatoxin B_1. There can be little doubt that the pyrazines are potent clastogenic agents.

Similarly, the tests with yeast revealed an increase in the number of aberrant colonies formed after treatment with each of the five pyrazine samples. However, no mitotic recombinants were observed among these aberrant colonies.

In contrast to the positive results obtained with the chromosome aberration test using a mammalian cell and the mitotic recombination assay in yeast, the widely applied *S. typhimurium* mutagenicity test gave negative results. We encountered a comparable situation with several reducing agents, including ascorbate, glutathione, cysteine and cysteamine (Stich *et al.* 1978). Caramelized sucrose, glucose, mannose and maltose also lacked the capacity to induce revertants in the plate test using *S. typhimurium* strain 98 and 100, whereas they were potent clastogenic agents elevating the frequencies of chromosome aberrations in CHO cells by 20 to 50-fold above those of non-exposed cells (Stich *et al.* 1980).

The results point to the necessity of using several test organisms and endpoints to avoid misleading conclusions. Such an approach is particularly required when untested chemicals with unknown reactive groups are examined for a potential mutagenic hazard.

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N-NITROSO COMPOUNDS FROM REACTIONS OF NITRITE WITH METHYLAMINE

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Abstract—*N*-Nitrosodimethylamine (NDMA) is formed when methylamine is reacted with acidic nitrite. Higher yields of NDMA are obtained at pH 5 than at pH 2; the reaction is catalysed by thiocyanate and by formaldehyde, and the effectiveness of these catalysts is also pH-dependent. *N*-Nitrosomethylmethoxymethylamine is also obtained when the reaction is carried out in the presence of formaldehyde.

INTRODUCTION

The possible formation of potentially carcinogenic or mutagenic *N*-nitrosodialkylamines (nitrosamines) from primary amines has received little recent attention in comparison to the extensive research programmes focused on secondary amines and, to a somewhat lesser extent, tertiary amines (Lijinsky, Keefer, Conrad & Van de Bogart, 1972; Walker, Bogovski & Griciute, 1976; Walker, Castegnaro, Griciute & Lyle, 1978).

Reports of the formation of *N*-nitrosodialkylamines during the nitrosation of primary amines extend over a period of more than 100 years (Fridman, Mukhametshin & Novikov, 1971; Warthesen, Scanlan, Bills & Libbey, 1975) but, in many cases, these reactions were considered to be minor aspects of the projects and, consequently, of relatively little importance.

Our interest in this area was stimulated by the observation of unexpectedly large numbers of products on a nitrosamine-specific chromatogram following reactions of primary amines with nitrite ions in the presence of thiocyanate ions (Tannenbaum, Wishnok, Hovis & Bishop, 1978). When *n*-butylamine was reacted with nitrite alone, small amounts of at least five such compounds were observed. When the reaction was carried out in the presence of the thiocyanate ion, the yields of all of these products were enhanced and a number of additional products appeared (Tannenbaum *et al.* 1978).

These results suggested that the nitrosation reactions of primary amines were potentially complex and that certain small molecules, such as thiocyanate or formaldehyde, which catalyse the nitrosation of secondary amines, might also catalyse, and participate in, similar reactions of primary amines (Fan & Tannenbaum, 1973; Keefer & Roller, 1973).

We have therefore examined the nitrosation reactions of a simpler and more environmentally significant amine, methylamine (Neurath, Dünger, Pein, Ambrosius & Schreiber, 1977). The reactions were carried out at various pHs and in the presence of thiocyanate and formaldehyde in order to assess how these variations may affect the formation of toxicologically significant compounds from primary amines.

EXPERIMENTAL

Commercial reagent-grade chemicals were used in most experiments. Purities were checked by gas chromatography (GC) with detection by flame-ionization, mass spectrometry, or thermal energy analysis (TEA; Fine, Rufeh & Gunther, 1973). Methylamine was used either as a 40% solution (Eastman Organics, Rochester, NY) or condensed from a lecture bottle (Matheson, Coleman & Bell, Norwood, OH). No dialkylamine impurities were detected at levels that might have interfered with the nitrosation experiments. *N*-nitrosomethylmethoxymethylamine was the generous gift of Dr. Peter Roller of the National Cancer Institute.

Gas chromatography was carried out on a Varian-Aerograph Model 200 using Carbowax 20 M/TPA (1/8 in. × 10 ft stainless steel; 10% on Chromosorb A 100/200 mesh; 2% KOH), OV-17 (1/8 in. × 10 ft stainless steel; 10% on Chromosorb G-HP 80/100 mesh; 2% KOH), or SP-1000 (1/8 in. × 6 ft stainless steel; 5% on Chromosorb G-HP 80/100; 2% KOH). Mass spectra were obtained on an Hitachi-Perkin-Elmer RMU-6E spectrometer.

In a typical experiment 50 ml aqueous $NaNO_2$ and 50 ml aqueous KSCN or formaldehyde (as diluted Formalin) were combined in a round-bottomed flask and the pH was adjusted with 25% HCl. Aqueous methylamine (50 ml) was added and the pH was readjusted (see tables for concentrations). Methylene chloride was added and the mixture stirred magnetically for 1 hr. (Recovery studies showed that reaction in a two-phase system was more effective than multiple extraction, at the end of a single-phase reaction alone). The layers were allowed to separate and the organic layer was removed. The water layer was extracted with 4 × 50 ml methylene chloride, and the

Table 1. *The effects of pH, thiocyanate and formaldehyde on formation of NDMA by the reaction of nitrite with methylamine*

pH of reaction mixture	Concn of formaldehyde (mM)	Concn of thiocyanate (mM)	NDMA formed (nM)
2	0	0	830
2	0	31	800
2	10	0	3800
5	0	0	1640
5	0	31	6940
5	10	0	5400

NDMA = *N*-Nitrosodimethylamine
In each case the reaction mixture contained 38 mM-methylamine and 18 mM-sodium nitrite.

combined extracts were dried over Na_2SO_4, concentrated to approximately 15 ml with a Kuderna-Danish evaporator, and then quantitatively transferred to 25 ml or 50 ml volumetric flasks. These final concentrates were analysed by GC–TEA.

RESULTS

The quantitative results are shown in Tables 1 and 2.

In agreement with earlier reports (Fridman *et al.* 1971; Warthesen *et al.* 1975) *N*-nitrosodimethylamine (NDMA) was formed in low yield during the reaction of methylamine with acidic nitrite. This reaction was enhanced by thiocyanate and by formaldehyde. At pH 5, with an approximately two-fold excess of amine over nitrite (38:18 mM), 10 mM-formaldehyde effected a three-fold increase in the yield of NDMA while 30 mM-thiocyanate increased the yield by about four-fold. At pH 2 the yield of NDMA in the uncatalysed reaction was only about half that at pH 5. At this lower pH, there was no catalysis by the thiocyanate ion but the yield of NDMA was increased more than four-fold by 10 mM-formaldehyde.

When the reaction was carried out in the presence of formaldehyde, the major volatile *N*-nitroso product was *N*-nitrosomethylmethoxymethylamine (NMMA, Fig. 1a) which is a moderately potent lung carcinogen in Sprague–Dawley rats (Weissler & Schmähl, 1976). This compound was formed in concentrations three to five times higher than those of NDMA. The methanol required for this reaction (Yanigada, Barsotti, Harrington & Swern, 1973) is apparently that which is added to the formalin as a preservative.The formation of both of these compounds increased with increasing formaldehyde concentrations (Table 2).

Table 2. *Effect of formaldehyde on the formation of NDMA and NMMA*

Concn of formaldehyde (mmol)	Final concn (μM)	
	NDMA	NMMA
2·5	0·6	3·2
5	6	18
7·5	9	33
10	14	39

NDMA = N-Nitrosodimethylamine
NMMA = *N*-Nitrosomethylmethoxymethylamine
Methylamine (0·2 M) and sodium nitrite (0·2 M) were reacted at pH 3 for 1 hr. *N*-Nitrosopyrrolidine was used as the internal GC standard.

The reaction products were identified as NDMA and NMMA by comparison of GC retention times and mass spectra with those of authentic samples of these compounds. Lower concentrations of a third TEA-positive compound are observed at longer retention times in the formaldehyde-catalysed reaction, but this product has not yet been characterized.

DISCUSSION

Our results confirm and extend the earlier observations that stable *N*-nitroso compounds can be formed during the nitrosation of primary amines (Fridman *et al.* 1971; Tannenbaum *et al.* 1978; Yanigada *et al.* 1973). Neither our results nor these earlier results demonstrate definitively that this behaviour is completely general for primary amines. There are, however, reasonable straightforward and nonspecific pathways for these transformations and they are probably common although perhaps not universal.

NDMA, for example, is probably formed by a mechanism analogous to that suggested by Warthesen and co-workers for the formation of *N*-nitrosopyrrolidine and *N*-nitrosodibutylamine from 1,4-diaminobutane and *n*-butylamine, respectively (Warthesen *et al.* 1975). In this sequence (Fig. 1b), a dialkylamine is formed, from the reaction of a diazonium ion (2) with a molecule of unreacted amine, and is subsequently nitrosated.

A route to NMMA (Fig. 1c), involving prior formation of methylmethoxymethylamine from 1,3,5-trimethylhexahydro-1,3,5-triazine (3) and methanol, has been suggested by Yanigada and coworkers (1973). The effects of thiocyanate, formaldehyde, and pH, all of which are known to affect the nitrosation of secondary amines (Fan & Tannenbaum, 1973; Keefer & Roller, 1973; Yanigada *et al.* 1973) are consistent with these hypotheses.

The environmental or physiological significance of these observations is difficult to assess. Direct exposure to dialkylnitrosamines *via* reactions of simple primary amines and nitrite is probably, in most situations, much less significant than that from the corresponding nitrosation of secondary amines since the yields are generally much lower in the former case than in the latter (Fridman *et al.* 1971; Mirvish, 1970; Tannenbaum *et al.* 1978; Warthesen *et al.* 1975). In foods, the total concentrations of primary amines appear to be somewhat higher than the total concentrations of secondary amines (Neurath *et al.* 1977), but the differences would probably not offset the lower conversions. The situation may not, however, be as straightforward as might be inferred from this simple comparison of relative yields with relative concentrations of reactants.

The carcinogenic effects of the dialkylnitrosamines, for example, are believed to arise *via* a metabolic

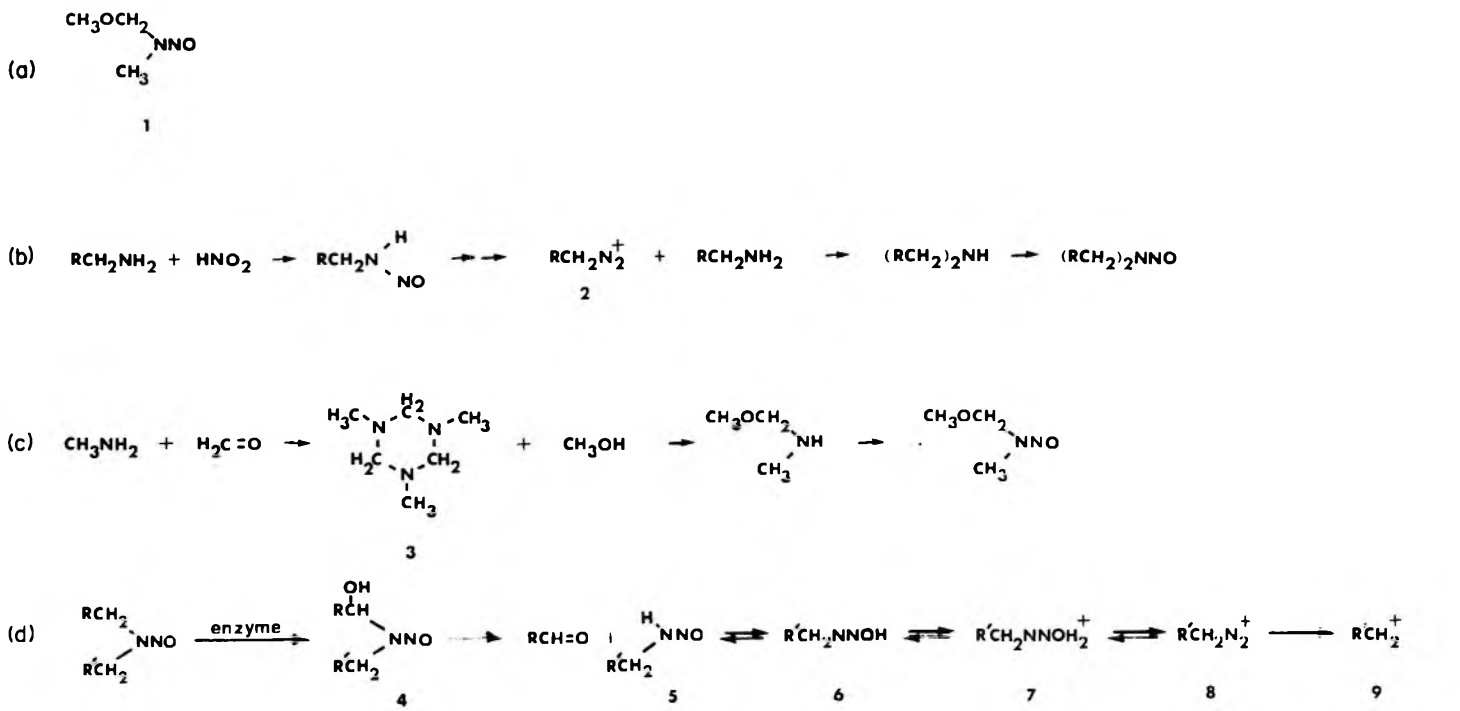

Fig. 1. (a) Structure of NMMA. (b) Probable mechanism of formation of NDMA. (c) Possible mechanism of formation of NMMA. (d) Possible sequence leading to the formation of a strong electrophile from a dialkylnitrosamine.

sequence initiated by enzymatic oxidation followed by a series of non-enzymatic steps leading to the formation of a strong electrophile (Fig. 1d). This electrophile is then believed to attack a nucleophilic site on a biological macromolecule, e.g., DNA, to initiate the tumorigenic sequence (Douglass, Kabacoff, Anderson & Cheng, 1978; Druckrey, Preussmann & Ivankovic, 1969; Magee & Barnes. 1967; Park, Wishnok & Archer, 1977; Scanlan, 1975).

The diazonium ion (8) or the carbonium ion (9) are often postulated as the 'ultimate' carcinogens in nitrosamine carcinogenesis (Douglass *et al.* 1978; Druckrey *et al.* 1969; Magee & Barnes, 1967; Scanlan, 1975), although there is evidence that, in some cases at least, earlier intermediates (e.g., 5 and 6 in Fig. 1d) are more likely to be involved (Park *et al.* 1977; Park, Archer & Wishnok, 1980).

These electrophiles, in any event, apparently arise from an intermediate *N*-nitroso *primary* amine (5). This implies, then, that the same set of intermediates can be formed directly *via* the nitrosation of primary amines, i.e., no metabolic activation would be necessary if this nitrosation occurred *in vivo*.

Thus, if nitrosation of a primary amine were to occur at or near a site of biological action, subsequent interactions with biological macromolecules could be indistinguishable from those of the metabolites of the corresponding *N*-nitrosodialkylamine. Although this condition of proximity would probably not often be met, it is conceivable that chronically high levels of nitrite and amine in, for example, the stomach, might contribute to the gastritis and metaplasia that precede tumours in some populations at high risk of gastric cancer (Tannenbaum, Archer, Wishnok, Correa, Cuello & Haenszel, 1977).

Two earlier results may be related to this hypothesis. Kriek & Emmelot (1964), showed that DNA could be methylated at the 7-position of guanine by *in vitro* incubation of the DNA with methylamine and nitrite. Hussein & Ehrenberg (1974) later noted that a mixture of methylamine and nitrite was mutagenic towards bacterial tester strains. Both of these observations are consistent with the behaviour expected from *N*-nitrosodimethylamine following metabolic activation and thus support current hypotheses of nitrosamine carcinogenicity and mutagenicity. These results, in addition, are consistent with the above suggestion that direct formation of the reactive primary nitrosamines may be of physiological significance.

In summary, primary amines and nitrite can react under a variety of conditions to form *N*-nitrosodialkylamines. The probable reaction pathways for these transformations, in addition, involve intermediates identical to those postulated to occur during the metabolic activation of dialkylnitrosamines to carcinogens. If human exposure to *N*-nitroso compounds is in fact partly due to *in vivo* nitrosation of amines, then it may be important to consider the contribution of primary amines when assessing the significance of these reactions.

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EFFECT OF ALCOHOLS ON NITROSAMINE FORMATION

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Abstract—Alcohols such as ethanol, methanol, *n*-propanol, isopropanol and sucrose in high concentrations inhibited the formation of *N*-nitrosodimethylamine or *N*-nitrosodiethylamine from sodium nitrite and the appropriate dialkylamine at pH 3·0 but enhanced the reaction at pH 5·0. Similarly, alcoholic drinks such as whisky, Japanese *sake* and wine inhibited nitrosamine formation at pH 3 and enhanced it at pH 5. Inhibition at pH 3 may have been due to the transformation of nitrite into inactive nitrite esters of the alcohols. The reasons for enhancement at pH 5 are obscure, since the nitrite esters could not nitrosate the amines nor catalytically activate the nitrosation at this pH. The effects of the alcohols on nitrosation were different from their effects on azo-dye formation, which was inhibited by ethanol and isopropanol at both pH 3 and pH 5.

INTRODUCTION

Nitrite is used as an intentional food additive and is readily produced by the bacterial and salivary reduction of nitrate present in vegetables (Ayanaba & Alexander, 1973). One of the important aspects of nitrite in foodstuffs is the production of potentially carcinogenic nitrosamines (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Magee & Barnes, 1967) by reaction with secondary amines under mildly acidic conditions such as those encountered in the stomach (Mirvish, 1970). Several endogenous compounds and others that may be added to foodstuffs are known to enhance nitrosamine formation. These include thiocyanate (Boyland, Nice & Williams, 1971), formaldehyde (Keefer & Roller. 1973), phenols such as occur in coffee (Challis & Bartlett, 1975), gallic acid (Walker, Pignatelli & Castegnaro, 1975), smoke phenols (Davies & McWeeny, 1977), nitrosophenol (Walker, Pignatelli & Castegnaro, 1979), a sesame-oil constituent (Kurechi, Kikugawa & Kato, 1979) and malondialdehyde (Kurechi, Kikugawa & Ozawa, 1980).

We have investigated the effects of alcohols on nitrosamine formation under acidic conditions. This paper deals with the effects of ethanol, methanol, *n*-propanol, isopropanol, sucrose and several commercial alcoholic drinks on the nitrosation reaction between sodium nitrite and dimethylamine or diethylamine.

EXPERIMENTAL

Materials. *N*-Nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) standards for gas chromatography were obtained from Wako Pure Chemical Industries Ltd., Osaka. Isoamyl nitrite (b.p. 90–100°C) was a product of Tokyo Kasei Kogyo Co. Ltd., Tokyo. Isopropyl nitrite (b.p. 40°C) was prepared according to the method of Levin & Hartung (1955). The commercial alcoholic drinks used were whisky (*Suntory Whisky* "*White*", ethanol content 40%, made by Suntory Ltd., Osaka), top-quality Japanese *sake* (Gekkeikan, ethanol content 16%, made by Okurashuzo Ltd., Kyoto) and wine (*Mercian*, ethanol content 14%, made by Sanraku-Ocean Ltd., Tokyo). Griess reagent was prepared by mixing, just before use, equal volumes of 1·0% (w/v) sulphanilic acid in 30% acetic acid (solution A) and 1·0% (w/v) 1-naphthylamine in 30% acetic acid (solution B). For the citrate buffer, sodium citrate solution in water was adjusted to the required pH with conc. HCl.

Analytical methods. A Yanaco Gas Chromatograph G80, equipped with a hydrogen flame ionization detector and a glass column (3 mm ID × 2 m) packed with polyethylene glycol 6000 (25%) on 80–100 mesh Chromosorb W AW, was used to determine the nitrosamines. The chromatograph was operated isothermally at 120°C (column temperature) and at 140°C (injector and detector temperature) with a nitrogen carrier-gas flow of 25 ml/min. The chromatograph was run with an internal standard, ethyl caproate. The amount of nitrosamine in the extract was determined by comparing the peak area of the samples (5 μl) with that of the authentic standard solution in chloroform (5 μl of 0·40 mg/ml). The formation of NDMA was confirmed by the use of a Hitachi Double Focusing Mass Spectrometer, RMU-7L after gas-chromatographic separation. Absorbance was measured with a Hitachi 101 spectrophotometer.

Effects of alcohols on nitrosamine formation. The effects were tested in three ways:

(a) To each of the alcohols in a range of concentrations in 50 ml 0·1 M-sodium citrate were added 3·0 ml 4 M-sodium nitrite and 3·0 ml M-dimethylamine (or diethylamine) hydrochloride; the mixtures were adjusted to the required pH with conc. HCl made up to 60 ml with water, and were incubated at 37°C for 3·5 hr in stoppered 100 ml flasks.

(b) To 40 ml 0·1 M-sodium citrate with or without isopropanol were added 5·0 ml M-dimethylamine and either 138 mg sodium nitrite, 178 mg isopropyl nitrite or 234 mg isoamyl nitrite; the mixtures were adjusted to the required pH, made up to 50 ml, and incubated at 37°C for 5 hr.

(c) To 50 ml of either whisky diluted (1:2) in water, or *sake* or wine were added 2·94 g sodium citrate dihydrate, 3·0 ml 4 M-sodium nitrite and 3·0 ml M-dimethylamine hydrochloride. The mixtures were adjusted to the required pH, made up to 60 ml, and incubated at 37°C for 3·5 hr.

The pH values of the reaction mixtures were generally unchanged during the reaction. From the reaction mixtures, 10-ml portions were removed and extracted with 40 ml chloroform in the presence of 2 g NaCl (extraction 1) or with 40 ml chloroform in the presence of 2 g NaCl and 5 ml 5 N-NaOH (extraction 2). The volumes of the chloroform extracts were measured and the extracts were subjected to gas-chromatographic analysis as soon as possible.

Effects of alcohols on diazotization. To 9·0 ml 0·2 M-citrate buffer (pH 3·0 and 5·0) were added 1·0 ml 40 mM-sodium nitrite and 4·0 or 10·0 ml ethanol or 4·0 ml isopropanol. The mixtures were made up to 20 ml with water and kept at room temperature for 3 hr. Portions of 0·10 ml were diluted to 10·0 ml with 2% acetic acid, and the solution was treated with 0·10 ml Griess reagent at room temperature for 15 min. The absorbances at 520 nm were measured.

RESULTS AND DISCUSSION

The effects of ethanol on the formation of NDMA by reaction of 0·2 M-sodium nitrite and 0·05 M-dimethylamine at 37°C for 3·5 hr at pH 3, 4 or 5 are summarized in Table 1. For each reaction mixture, gas-chromatographic determination of the nitrosamine content showed no significant difference between the two chloroform-extraction methods used (direct extraction of the acidic reaction mixture or extraction after alkalination of the mixture).

NDMA formation was considerably influenced by ethanol. At pH 3, concentrations of 17 and 42% ethanol inhibited NDMA formation by 77 and over 90%, respectively, when compared with the control system. At pH 4, the formation was again reduced by ethanol but the effect was less marked. At pH 5, NDMA formation was increased to 260 and 800% by 17 and 42% ethanol, respectively. This increase, demonstrated by gas chromatography, was confirmed by mass spectrometry. The dependence of nitrosamine formation on pH was reversed in the ethanol-containing systems compared with that in the controls. Thus, the amount of nitrosamine formed decreased with increasing pH in the controls and increased with increasing pH in the ethanol-containing systems. The NDMA formation at pH 5 in a solution containing 42% ethanol was close to that at pH 3 in the control. Ethanol had a significant effect in concentrations above 10% (data not tabulated), but no significant effects were noted at concentrations below 5%.

The other alcohols, methanol, *n*-propanol and isopropanol as well as sucrose, which has several alcoholic hydroxyl groups, had similar effects on NDMA formation when tested under the same conditions (Table 2). Thus 17% methanol, *n*-propanol or isopropanol inhibited nitrosation at pH 3 by more than 80%, and enhanced it at pH 5 by 120–300%. Sucrose in high concentrations (21 and 42%) also increased NDMA formation at pH 5.

The reaction of 0·2 M-sodium nitrite with 0·05 M-diethylamine was also influenced by ethanol (Table 3). NDEA formation was inhibited by 17% ethanol by more than 90% at pH 3, and was elevated to 280% at pH 5.

It has been well documented that alcohols are readily converted into their nitrite esters by acid-catalysed reaction with sodium nitrite (Coffey, 1965). In the present experiments, a considerable evolution of

Table 1. *Effects of ethanol on NDMA formation from 0·2 M-nitrite and 0·051 M-dimethylamine in a 3·5-hr incubation at 37°C*

		NDMA formation		
		Acidic extraction*	Alkaline extraction†	
pH	Ethanol concn (% v/v)	Ratio‡	Ratio‡	As % of pH-specific control value
3·0	0	1·00	1·03	100
	17§	—	0·24	23
	42§	0·06	0·09	9
4·0	0	0·57	0·57	100
	17	—	0·49	86
	42	0·30	0·33	58
5·0	0	0·11	0·11	100
	17	—	0·29	264
	42	0·84‖	0·88	800

NDMA = *N*-Nitrosodimethylamine

* Direct extraction of reaction mixture with chloroform.

† Extraction of reaction mixture with chloroform after addition of NaOH.

‡ All values are expressed as ratios of the nitrosamine concentration (0·037–0·042 M) reached in the control (ethanol-free) reaction at pH 3, as determined after direct extraction at acid pH.

§ Considerable evolution of gas.

‖ NDMA confirmed by mass spectroscopy.

Table 2. *Effects of methanol, n-propanol, isopropanol and sucrose on NDMA formation from 0·2 M-nitrite and 0·051 M-dimethylamine in a 3·5-hr incubation at 37°C*

pH	Alcohol (with % v/v concn)	NDMA formation*	
		Ratio based on pH-3 control value	As % of pH- specific control value
3·0	None (control)	1·00	100
	Methanol (17)†	0·03	3
	n-Propanol (17)‡	0·02	2
	Isopropanol (17)	0·15	15
5·0	None (control)	0·11	100
	Methanol (17)	0·14	127
	n-Propanol (17)	0·23	209
	Isopropanol (17)	0·33	300
	Sucrose (21§)	0·15	132
	(42§)	0·17	158

NDMA = *N*-Nitrosodimethylamine
* Direct chloroform extraction of acidic reaction mixture.
† Considerable evolution of gas.
‡ Separation of yellow oil.
§ % w/v.

gas from the pH 3 reaction mixtures containing ethanol and methanol (Tables 1–3) may have been due to the formation of the highly volatile nitrite esters, ethyl nitrite b.p. 18°C and methyl nitrite b.p. −16°C (Coffey, 1965). The reaction mixture containing *n*-propanol (Table 2) separated a yellow oil at pH 3, probably as a result of the formation of the nitrite ester, which is of low volatility (b.p. 49°C; Coffey, 1965) and low solubility. The loss of nitrite either as a gas or insoluble oil from the pH 3 reaction mixtures could result in a decrease in available nitrite for the nitrosation of dimethylamine or diethylamine. The presence of isopropanol in the reaction mixture was not accompanied by any appreciable formation of gas or oil at pH 3, but nitrosation was inhibited by this as by the other alcohols (Table 2). No gas or oil formation occurred in any of the reactions at pH 5, in which the presence of the alcohols enhanced nitrosamine formation.

To study further the influence of alcohols on the nitrosation, the effects of isopropanol, isopropyl nitrite and isoamyl nitrite were compared in the reaction of 0·04 M-sodium nitrite with 0·10 M-dimethylamine at 37°C for 5 hr (Table 4). The reaction was again inhibited at pH 3 and enhanced at pH 5 by 20% isopropanol. Neither inhibition nor stimulation occurred when isopropanol was added only just before the extraction of the mixture resulting from reaction of sodium nitrite and dimethylamine alone. The nitrosation potential of both isopropyl nitrite and isoamyl nitrite was negligible at pH 3 and 5 and in the presence or absence of isopropanol. Thus, inhibition of nitrosamine formation by isopropanol at pH 3 could be ascribed to the conversion of nitrite to its ester. When sodium nitrite was reacted in the presence of isopropyl nitrite or isoamyl nitrite, the level of nitrosamine formation was the same as that occurring in the absence of the esters, demonstrating that these nitrite esters did not influence the nitrosation of the amine by sodium nitrite at either pH 3 or pH 5. Although some degree of ester formation with consequent loss of active nitrite may occur at pH 5, the nitrosamine formation was markedly stimulated at this pH. The reasons for this stimulation have not been elucidated yet. The pattern of the effects of alcohols on nitrosation by nitrite, namely inhibition at pH 3 and stimulation at pH 5, resembles that of gallic acid (Walker *et al.* 1975) and of malondialdehyde (Kurechi *et al.* 1980).

Table 3. *Effects of ethanol on NDEA formation from 0·2 M-nitrite and 0·051 M-diethylamine in a 3·5-hr incubation at 37°C*

pH	Ethanol concn (% v/v)	NDEA formation*	
		Ratio based on pH-3 control value	As % of pH- specific control value
3·0	0	1·00†	100
	17‡	0·08	8
5·0	0	0·09	100
	17	0·25	278

NDEA = *N*-Nitrosodiethylamine
* Direct chloroform extraction of reaction mixture.
† Concn of NDEA in this reaction was 7·1 mM.
‡ Considerable evolution of gas.

Table 4. *Effects of isopropanol, isopropyl nitrite and isoamyl nitrite on NDMA formation from nitrite and dimethylamine in a 5·0-hr incubation at 37°C*

	Reaction mixture					NDMA formation*	
pH	Sodium nitrite (mM)	Isopropanol (% v/v)	Isopropyl nitrite (mM)	Isoamyl nitrite (mM)	DMA (mM)	Ratio based on pH-3 control value	As % of pH- specific control value
3·0	40	—	—	—	100	1·00†‡	100
	40	20	—	—	100	0·26	26
	—	—	40	—	100	0	0
	—	20	40	—	100	0	0
	—	—	—	40	100	0	0
	40	—	40	—	100	1·03	103
	40	—	—	40	100	1·07	107
5·0	40	—	—	—	100	0·10‡	100
	40	20	—	—	100	0·29	290
	—	—	40	—	100	0	0
	—	20	40	—	100	0	0
	—	—	—	40	100	0	0
	40	—	40	—	100	0·10	100
	40	—	—	40	100	0·11	110

NDMA = *N*-Nitrosodimethylamine DMA = Dimethylamine

* Direct chloroform extraction of acidic reaction mixture.

† Concn of NDMA in this reaction was 3·8–4·2 mM.

‡ NDMA concn was unaffected by addition of 20% isopropanol to the reaction mixture just before the extraction.

Pignatelli, Castegnaro & Walker (1976) described the catalytic effect of ethanol on the nitrosation of diethylamine in the acidic range. Yamamoto, Yamada & Tanimura (1979) have claimed that this effect was possibly due to the stimulation of NDEA formation by alkalination of the reaction mixture. They based this view on their experiments demonstrating that ethyl nitrite formed in the acidic reaction mixture could readily nitrosate diethylamine in the relatively strongly alkaline range. In the present experiment, there were no significant differences in nitrosamine formation between the extractions from the mixtures of high and low pH (Table 1). This discrepancy between the two experiments may have been due to the differing reaction conditions, such as the concentration of ethanol, reaction time, reaction temperature and so on. In most of the experiments described here, however, the nitrosamines were extracted directly from the reaction mixtures with chloroform in order to avoid this kind of troublesome influence.

The effects of commercial alcoholic drinks, such as whisky (40% ethanol), Japanese *sake* (16% ethanol) and wine (14% ethanol) on the formation of the nitrosamine from 0·2 M-sodium nitrite and 0·05 M-dimethylamine are shown in Table 5. The concentrations of ethanol in the reaction mixtures were adjusted to between 11 and 17%. These concentrations inhibited nitrosamine formation at pH 3, and enhanced the formation at pH 5. Although the effects of these alcoholic drinks could be ascribed not only to ethanol but also to other contaminants, the effects were comparable to those of pure ethanol. The finding that alcoholic drinks in high concentrations enhanced nitrosamine formation at pH 5 may be of some relevance to the frequent nitrosamine contamination of alcoholic drinks, such as beer, whisky and brandy. These

Table 5. *Effects of alcoholic drinks on NDMA formation from 0·2 M-nitrite and 0·051 M-dimethylamine in a 3·5-hr incubation at 37°C*

			NDMA formation*	
pH	Alcoholic drink	Ethanol (%) in reaction mixture	Ratio based on pH-3 control value	As % of pH- specific control value
3·0	None	0	1·00	100
	Whisky	16·7	0·41	41
	Sake	13·3	0·11	11
5·0	None	0	0·11	100
	Whisky	16·7	0·27	245
	Sake	13·3	0·23	209
	Wine	11·7	0·21	191

NDMA = *N*-Nitrosodimethylamine

* Direct chloroform extraction of acidic reaction mixture.

Table 6. *Azo-dye formation in Griess reagent following treatment of 2·0 mM-nitrite with ethanol or isopropanol for 3·0 hr at room temperature*

Treatment		
pH	Alcohol (with % v/v concn)	Ratio of absorbance at 520 nm
3·0	None	1·00
	Ethanol (20)	0·49
	(50)	0·26
	Isopropanol (20)	0·69
5·0	None	1·00
	Ethanol (20)	0·85
	(50)	0·72
	Isopropanol (20)	0·91

data demonstrated the readiness with which nitrosamines may be formed under mildly acidic conditions in foodstuffs or drinks that contain alcohols in high concentration and in which both nitrite and secondary amines are also present. Furthermore, nitrosamines may be readily produced in drugs, cosmetics and industrial materials containing alcoholic substances.

In order to compare the effects of alcohols on the nitrosation of secondary amines with their effects on azo-dye formation, sodium nitrite treated with an alcohol was estimated by the Griess reagent, which is commonly used for the determination of nitrite in foodstuffs (Horwitz, 1975) and which reacts with nitrite at pH 2–3 to form a red dye *via* diazotization of sulphanilic acid and subsequent coupling with 1-naphthylamine. Sodium nitrite (2 mM) was treated with 20 or 50% ethanol or with 20% isopropanol at pH 3 and 5 at room temperature for 3 hr, and the nitrite was then determined with Griess reagent. The amount of nitrite available for azo-dye production was reduced at both pH 3 and pH 5 (Table 6). Since a nitrite ester, such as isopropyl nitrite, had a potency that was only about half that of the nitrite ion under the conditions of these experiments, these inhibitory effects may be ascribed to formation of the less active nitrite ester of the alcohol. Thus, the effects of alcohols on azo-dye formation were not comparable to those on secondary amine nitrosation. It is apparent that the potential of nitrite for reacting with the Griess reagent to form an azo-dye is not an accurate reflection of the nitrite available for nitrosation in foodstuffs and drinks containing alcohols.

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CHANGES IN THE NITRATE AND NITRITE CONTENTS OF FRESH VEGETABLES DURING CULTIVATION AND POST-HARVEST STORAGE

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Abstract—Nitrate and nitrite levels in both germinating seeds and growing vegetables were determined for the following vegetables: short-petioled cabbage (*Brassica chinensis*), long-footed cabbage (*Brassica chinensis* var. *communis*), field mustard (*Brassica campestris*), broad-leaf mustard (*Brassica juncea* var. *rugosa*) and water convolvulus (*Ipomoea aquatica* Forsk). The seeds contained 16–32 ppm nitrogen in the form of nitrate and 0·3–0·8 ppm nitrogen in the form of nitrite. The nitrate nitrogen concentrations of the growing vegetables varied between 300 and 1200 ppm depending on species and their nitrite nitrogen contents were about 0·3–3 ppm. The nitrogen fertilizer urea seemed to induce remarkable nitrate accumulation in the growing vegetables. The influence of storage at different temperatures on the nitrate and nitrite contents of fresh and homogenized vegetables was studied. During the first few days storage temperatures of −10 or 2°C little change in the nitrate and nitrite concentrations was found. However during the first few days of storage at 26 or 32°C the nitrite concentration increased considerably while the nitrate concentration decreased. Nitrate reductase and nitrite reductase activities in the vegetables were estimated at various stages of growth but no significant changes in activity were observed.

In view of the effect of dietary nitrate on salivary nitrite formation and on the endogenous formation of carcinogenic *N*-nitroso compounds, we suggest that more effort should be put into developing methods of cultivating low-nitrate vegetables.

INTRODUCTION

The occurrence in foods of nitrate, nitrite and *N*-nitroso compounds has recently become a matter of great concern because of the toxic and carcinogenic nature of these compounds (Shank, 1975; Weisburger & Raineri, 1975; Wogan & Tannenbaum, 1975; Wolff & Wasserman, 1972). Concern was previously focused on the relationship of nitrate and nitrite in the diet to infant methaemoglobinaemia (Phillips, 1971). More recently, nitrite has been shown to react with secondary or tertiary amines to form carcinogenic *N*-nitroso compounds, and investigations have been undertaken to examine the possible role of nitrate and nitrite in the incidence of human cancer (Swann, 1975).

Some vegetables, such as spinach and beets, can accumulate high levels of nitrate, which may be reduced to nitrite during storage after preparation (Aworh, Brecht & Minotti, 1978; Heisler, Siciliano, Krulick, Feinberg & Schwartz, 1974). It has been estimated that four-fifths of US dietary nitrate intake is from vegetables, and less than one-sixth from cured meat and that two-thirds of the nitrite entering the average stomach originates in saliva and slightly less than one-third comes from cured meats; other sources of nitrite are not significant (White, 1975).

The effect of nitrate intake on the formation of salivary nitrite has been studied in a series of individuals using celery juice as the source of nitrate (Tannenbaum, Weisman & Fett, 1976). The extent of nitrite formation is related to the quantity of nitrate, to the concentration of the nitrate source and to the oral microflora. The possible relevance of these findings to the endogenous formation of *N*-nitroso compounds in the gastro-intestinal tract has been discussed (Spiegelhalder, Eisenbrand & Preussmann, 1976; Tannenbaum, Sinskey, Weisman & Bishop, 1974).

The threat of nitrate toxicity to man from ingestion of vegetables has stimulated widespread interest and research in nitrate accumulation by vegetables crops. The objective of our study was to determine the progressive changes in the endogenous nitrate and nitrite levels in germinating and growing vegetables such as short-petioled cabbage, long-footed cabbage, field mustard, broad-leaf mustard and water convolvulus. The influence of post-harvest temperature and storage duration on nitrate and nitrite levels in these vegetables was also studied.

EXPERIMENTAL

Cultivation and handling of vegetables. The following vegetables which are currently consumed by most Chinese people here in Taiwan were selected for this study: short-petioled cabbage (*Brassica chinensis*), long-footed cabbage (*Brassica chinensis* var. *communis*), field mustard (*Brassica campestris*), broad-leaf mustard (*Brassica juncea* var *rugosa*) and water convolvulus (*Ipomoea aquatica* Forsk). The vegetables were grown in the summer of 1979 at the University Garden located at Jen-Ai District, Taipei. Nitrogen fertilizer comprising 2·8 kg N/are as urea, 0·57 kg P/are as P_2O_5 and 1·05 kg K/are as KCl was broadcast at planting. Additional urea (0·9 kg N/are ×3) was side-dressed 5, 9 and 15 days after planting the seeds.

The plants were harvested weekly after planting. Samples (100 g) of intact vegetable leaves with petioles attached were taken immediately after harvest for subsequent chemical analyses and enzymatic assays.

In the 3-day storage experiments, samples of each of the five species of vegetables were placed in plastic bags and held at −10, 2, 15 or 26°C for 0, 1, 2 or 3 days. About 20 g of vegetable leaves were removed daily for biochemical analyses.

Determination of nitrate and nitrite. Vegetable samples (20 g) were washed with 30 ml of distilled water, and the retained water was absorbed using Whatmann no. 1 filter paper. The samples were then homogenized with 80 ml of 0·9% NaCl in a Waring blendor. Ten ml of the homogenate was taken and heated in a boiling water bath for 10 min. The resulting mixture was passed through an alumina column (5 cm × 10 mm) to remove debris and pigments, the column was then washed with distilled water until 25 ml of eluate was obtained. A 1-ml aliquot was then taken for the determination of nitrite and a 10-ml aliquot was used for the nitrate estimation.

Nitrite concentration was determined by the classic Griess reaction with sulphanilic acid and α-naphthylamine in diluted acetic acid medium (AOAC, 1975). Nitrate concentration was determined by reduction to nitrite with cadmium column (12 cm × 10 mm) followed by the nitrite analysis (AOAC, 1975).

Nitrate and nitrite concentrations in the seeds of vegetables were similarly determined. Changes in nitrate and nitrite concentrations during the germination of these seeds were also studied. At each time interval, the seeds (at days 0, 1 and 2) or seedlings (at day 5) were weighed and homogenized with 0·9% NaCl to make 20% homogenates.

Changes in nitrate and nitrite concentrations during storage of fresh long-footed cabbage were also studied in a 20% homogenized sample. The cabbage samples were homogenized aseptically with 0·8 vol. of 0·9% NaCl. Aliquots (10 ml) of the homogenates were placed in a series of sterilized 15-ml test tubes and incubated at 2 or 32°C. Samples were removed daily for nitrate and nitrite determinations.

Estimation of nitrate reductase and nitrite reductase. It has been demonstrated that the activity of nitrate reductase is selectively inhibited by sodium azide (1 mM) which exerts a negligible effect on the activity of nitrite reductase (Cresswell, Hageman, Hewitt & Hucklesby, 1960; Evans & Nason, 1953). Therefore, the activities of these two enzymes in vegetable homogenates were estimated differentially using this inhibitor. The reactions were carried out aerobically in three 25-ml test tubes. Each tube contained 2 ml sodium nitrate solution (100 ppm), 2 ml sodium nitrite solution (10 ppm) 10 ml vegetable homogenate (20%). Sodium azide solution (1 ml) was added to tube 3 to make a final concentration of 1 mM and 1 ml of distilled water was added to each of tubes 1 and 2. Tube 1 was heated in a boiling water bath for 15 min to stop the enzyme reaction as soon as the vegetable homogenate was added and mixed. All the tubes were incubated at 37°C for 30 min. Tubes 2 and 3 were then heated similarly to stop the enzyme reaction. The reaction mixture in each tube was passed through an alumina column and the nitrite concentration was determined as described above. Suppose the nitrite concentrations in tubes 1, 2 and 3 are found to be D_1, D_2 and D_3 μg nitrite-N/g vegetable/30 min, respectively, the activities of nitrate reductase and nitrite reductase can be calculated as follows:

nitrate reductase activity
$= D_2 - D_3$ (μg nitrite-N/g/30 min)
nitrite reductase activity
$= D_1 - D_3$ (μg nitrite-N/g/30 min).

RESULTS

Nitrate and nitrite contents in the germinating seeds

The contents of nitrate and nitrite in the germinating seeds of four vegetables were determined periodically. The results showed that an appreciable amount of nitrate-N was present in the original seeds (Table 1). The level of nitrate was reduced dramatically as soon as germination was initiated. Nitrite-N concentrations in the seeds were found to be low and no significant change was detected during the whole period of germination. Levels were not determined in water convolvulus seeds as the testa is too tough for homogenization.

Nitrate and nitrite contents in the growing vegetables

Nitrate-N concentrations in five growing vegetables

Table 1. *Changes in nitrate and nitrite contents in germinating vegetable seeds*

Vegetable seed	Weight of ten seeds (g)	Germination day...	Concn of nitrate-N in seeds (ppm) 0	1	2	5*	Concn of nitrite-N in seeds (ppm) 0	1	2	5*
Short-petioled cabbage (*Brassica chinensis*)	22·2		25·2	1·1	1·0	0	0·6	0·4	0·3	0·2
Long-footed cabbage (*Brassica chinensis* var. *communis*)	19·4		15·7	1·8	0	0·2	0·3	0·5	0·7	0·9
Field mustard (*Brassica campestris*)	21·5		28·1	5·1	2·2	0·6	0·8	0·9	0·7	0·7
Broad-leaf mustard (*Brassica juncea* var. *rugosa*)	10·7		31·6	3·4	0·2	0	0·4	0·4	0·4	1·3

Germination was initiated by placing the vegetable seeds with a suitable amount of distilled water in a sterilized petri dish. At each time interval, approximately 10 g of seeds were removed for nitrate and nitrite determination. Data are the means of three determinations.

*On day 5 seedlings (20 g) were collected and analysed.

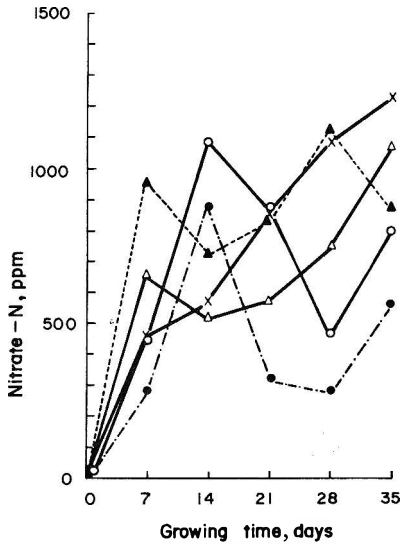

Fig. 1. Changes in the nitrate-N contents of the growing vegetables from the initiation of germination. The species of vegetables are: short-petioled cabbage (—△—), long-footed cabbage (— × —), field mustard (--○—), broad-leaf mustard (--▲--) and water convolvulus (-·-●-·-). Data are the means for three determinations.

were determined weekly and the results are illustrated in Fig. 1. A fast increase in nitrate-N content with an initial peak on day 7 was observed in both short-petioled cabbage and broad-leaf mustard, while a slightly slower increase reaching a peak at day 14 was detected in both field mustard and water convolvulus. A second phase of increase in nitrate content was observed in all these four vegetables. During the course of a 35-day period, a steady increase of nitrate-N concentration was observed in long-footed cabbage.

Nitrite-N concentrations in these growing vegetables are summarized in Fig. 2. A very sharp increase and then a decrease in nitrite-N content was observed in both field mustard and water convolvulus, while no significant change was detected in the other three species.

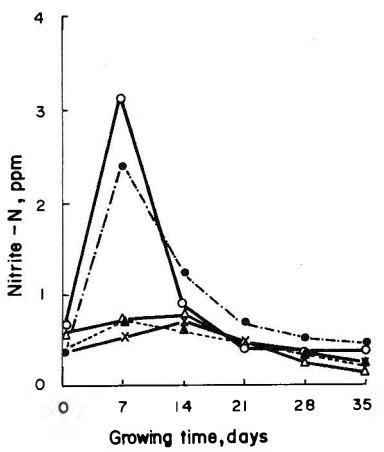

Fig. 2. Changes in the nitrite-N contents of the growing vegetables. The species of vegetables are short-petioled cabbage (—△—), long-footed cabbage (— × —), field mustard (—○—), broad-leaf mustard (--▲--) and water convolvulus (-·-●-·-). Data are the means for three determinations.

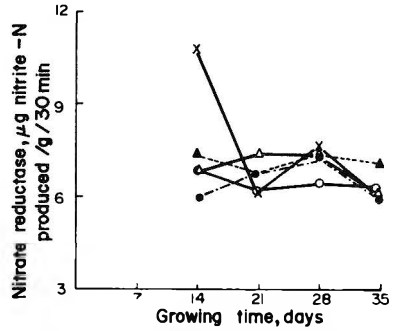

Fig. 3. Changes in the activity of nitrate reductase in the growing vegetables. The species of vegetables are short-petioled cabbage (—△—), long-footed cabbage (— × —), field mustard (—○—), broad-leaf mustard (--▲--) and water convolulus (-·-●-·-). Data are the means for three estimations.

Nitrate reductase and nitrite reductase in the growing vegetables

The activity of nitrate reductase in the growing vegetables is summarized in Fig. 3. The enzyme activity in long-footed cabbage was high at day 14, but decreased later on. The enzyme activity in the other vegetables was kept at a fairly constant level during the growing period.

The activity of nitrite reductase in the growing vegetables is illustrated in Fig. 4. Nitrite reductase activity was high in the early period but decreased during the later stages.

Nitrate and nitrite contents in the fresh vegetables during storage

The changes in nitrate-N in the stored vegetables are summarized in Table 2. It appeared that slightly less changes in nitrate-N in these vegetables occurred during the 3 days of storage at −10 and 2°C, but nitrate-N decreased fairly rapidly if the vegetables

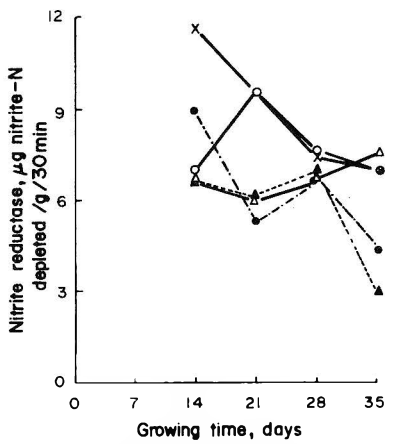

Fig. 4. Changes in the activity of nitrite reductase in the growing vegetables. The species of vegetables are short-petioled cabbage (—△—), long-footed cabbage (— × —), field mustard (—○—), broad-leaf mustard (--▲--) and water convolvulus (-·-●-·-). Data are the means for three estimations.

Table 2. *Effects of storage on nitrate levels in fresh vegetables*

Vegetable	Storage day	Storage temperature (°C)...	Nitrate-N concn (ppm)			
			−10	2	15	26
Short-petioled cabbage	0		1074	1074	1074	1074
(*Brassica chinensis*)	1		938	766	1083	1073
	2		834	1029	1250	1250
	3		874	979	896	606
Long-footed cabbage	0		1220	1220	1220	1220
(*Brassica chinensis* var. *communis*)	1		818	1210	975	1129
	2		864	1033	1045	1250
	3		754	1242	889	713
Field mustard	0		808	808	808	808
(*Brassica campestris*)	1		496	749	539	1235
	2		1043	1321	1183	1001
	3		714	639	1548	431
Broad-leaf mustard	0		877	877	877	877
(*Brassica juncea* var. *rugosa*)	1		487	1048	1095	573
	2		549	1120	1453	859
	3		1252	19	744	1006
Water convolvulus	0		783	783	783	783
(*Ipomoea aquatica* Forsk)	1		515	526	633	540
	2		1160	875	760	713
	3		429	437	624	553

Data are the means of three determinations.

were stored at 26°C. During the first 3 days of storage at 26°C, nitrate-N in short-petioled cabbage, long-footed cabbage and field mustard were reduced to approximately 50–60% of their initial levels. A transient increase in nitrate-N was detected in most vegetables after 2 days of storage; in some cases, the increase period extended to 3 days and then a decrease phase followed.

The levels of nitrite-N in vegetables were fairly constant when they were stored at −10, 2 and 15°C for 3 days (Table 3). The concentration of nitrite-N began increasing after 3 days of storage at 26°C while nitrate-N decreased. This indicates that conversion of nitrate to nitrite occurs during this period.

Vegetables stored at 26°C deteriorated rapidly and were considered unmarketable after 2 days and unusable after 3 days.

Nitrate reductase and nitrite reductase in the vegetables during storage

The activities of nitrate reductase and nitrite reductase in the stored vegetables were estimated and are

Table 3. *Effect of storage on nitrite levels in fresh vegetables*

Vegetable	Storage day	Storage temperature (°C)...	Nitrite-N concn (ppm)			
			−10	2	15	26
Short-petioled cabbage	0		0·2	0·2	0·2	0·2
(*Brassica chinensis*)	1		0·4	0·4	0·2	0·2
	2		0·3	0·4	0·3	2·6
	3		0·4	0·3	0·3	125·6
Long-footed cabbage	0		0·2	0·2	0·2	0·2
(*Brassica chinensis* var. *communis*)	1		0·3	0·4	0·3	0·2
	2		0·3	0·3	0·2	2·4
	3		0·3	0·2	0·2	132·9
Field mustard	0		0·3	0·3	0·3	0·3
(*Brassica campestris*)	1		0·4	0·4	0·2	0·2
	2		0·3	0·2	0·2	1·0
	3		0·4	0·2	0·2	138·6
Broad-leaf mustard	0		0·3	0·3	0·3	0·3
(*Brassica juncea* var. *rugosa*)	1		0·4	0·6	0·3	0·3
	2		0·4	0·3	0·2	3·6
	3		0·2	0·2	0·3	12·1
Water convolvulus	0		0·3	0·3	0·3	0·3
(*Ipomoea aquatica* Forsk)	1		0·3	0·3	0·2	0·4
	2		0·3	0·3	0·4	0·5
	3		0·5	0·4	0·3	0·7

Data are the means of three determinations.

Table 4. *Effects of storage on the activity of nitrate reductase in vegetables*

Vegetable	Storage day	Storage temperature (°C)...	Nitrate reductase −10	2	15	26
Short-petioled cabbage	0		7·9	7·9	7·9	7·9
(*Brassica chinensis*)	1		6·6	6·3	5·8	6·6
	2		4·6	—	6·7	7·1
	3		5·9	5·8	5·6	15·9
Long-footed cabbage	0		7·0	7·0	7·0	7·0
(*Brassica chinensis* var. *communis*)	1		6·3	6·3	6·3	5·3
	2		6·3	6·2	6·7	6·4
	3		5·9	5·7	5·9	10·6
Field mustard	0		7·0	7·0	7·0	7·0
(*Brassica campestris*)	1		6·4	6·1	6·7	6·2
	2		6·3	6·2	6·7	7·5
	3		5·8	5·8	3·9	13·1
Broad-leaf mustard	0		2·8	2·8	2·8	2·8
(*Brassica juncea* var. *rugosa*)	1		4·9	6·3	6·3	6·6
	2		5·8	6·2	—	7·3
	3		4·5	5·2	5·6	31·1
Water convolvulus	0		4·4	4·4	4·4	4·4
(*Ipomoea aquatica* Forsk)	1		4·6	6·6	6·1	4·9
	2		5·1	4·3	5·1	4·9
	3		4·7	4·9	5·6	6·4

The enzyme activity is expressed as μg of nitrite-N formed/g of vegetable/30 min. Values are the means of three determinations.

summarized in Tables 4 and 5, respectively. Storage at −10, 2 and 15°C did not exert significant effect on the activities of these two enzymes, whereas storage at 26°C provoked a remarkable enhancing effect on the activities of these enzymes. The reason for this enhancement will be discussed later.

Nitrate and nitrite levels in the vegetable homogenates during storage

The nitrate-N contents of the vegetables were found to vary considerably from sample to sample. In order to overcome this problem in sampling vegetables for storage study, a large batch of long-footed cabbage was homogenized aseptically and used to study post-harvest storage. The nitrate and nitrite contents in the homogenates stored at 2 or 32°C were determined daily. The results are depicted in Fig. 5. The patterns of alterations in the levels of nitrate and nitrite were quite different at the two different temperatures. Nitrate-N concentration decreased rapidly in the

Table 5. *Effect of storage on the activity of nitrite reductase in vegetables*

Vegetable	Storage day	Storage temperature (°C)...	Nitrite reductase −10	2	15	26
Short-petioled cabbage	0		6·3	6·3	6·3	6·3
(*Brassica chinensis*)	1		6·2	6·2	6·3	6·9
	2		6·2	—	6·4	7·3
	3		5·6	5·3	4·2	14·0
Long-footed cabbage	0		6·1	6·1	6·1	6·1
(*Brassica chinensis* var. *communis*)	1		6·7	6·4	6·2	6·9
	2		5·6	6·3	6·4	6·6
	3		5·3	5·4	5·6	16·9
Field mustard	0		7·4	7·4	7·4	7·4
(*Brassica campestris*)	1		6·4	8·5	6·4	6·5
	2		5·9	6·3	6·3	8·0
	3		5·6	5·9	5·6	56·2
Broad-leaf mustard	0		0·5	0·5	0·5	0·5
(*Brassica juncea* var. *rugosa*)	1		1·4	6·1	5·9	6·3
	2		5·2	6·2	—	7·5
	3		4·6	5·1	5·4	21·3
Water convolvulus	0		6·1	6·1	6·1	6·1
(*Ipomoea aquatica* Forsk)	1		1·2	6·8	5·9	5·3
	2		4·7	5·4	5·5	5·1
	3		5·5	5·8	5·8	4·8

The enzyme activity is expressed as μg nitrite-N depleted/g of vegetable/30 min. Va ues are the means of three determinations.

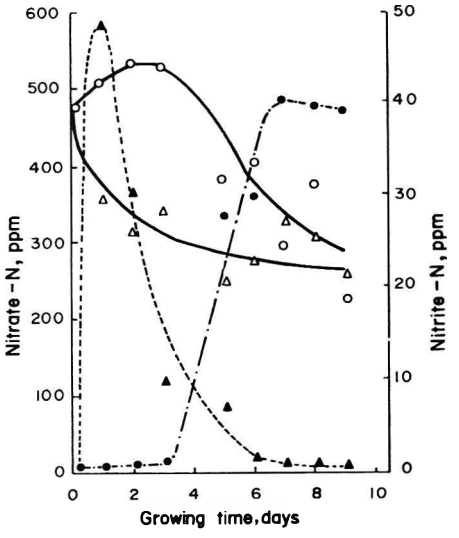

Fig. 5. Effect of storage at 2 and 32°C on the concentration of nitrate-N and nitrite-N in homogenized long-footed cabbage. Nitrate-N at 2°C (—○—), nitrate-N at 32°C (—△—), nitrite-N at 2°C (- - -●- - -) and nitrite at 32°C (- -▲- -).

samples stored at 32°C, but increased slowly at 2°C during the first 3 days. Subsequently the nitrate-N in the vegetable homogenate stored at 2°C gradually decreased and that stored at 32°C decreased even more slowly. Nitrite-N concentrations in the homogenates stored at 2°C were fairly constant during the first 3 days of storage, and then increased extremely rapidly to reach a plateau of 40 ppm by the 7th day. On the other hand, nitrite-N in the homogenates stored at 32°C increased rapidly and reached its peak during the first day of storage and then decreased progressively to nearly zero by the sixth day (Fig. 5).

DISCUSSION

Factors affecting the level of nitrate in vegetables

Nitrate accumulation in vegetables is a natural phenomenon resulting from uptake of the nitrate ion in excess of its reduction and subsequent assimilation. Maynard, Barker, Minotti & Peck (1976) considered that accumulation of nitrate is dependent on and related to the genetic makeup of the vegetable, the nitrate-supplying power of the soil, and the growing conditions.

Nitrosomonas and nitrobacter are generally considered to be widely distributed and agriculturally important in soils. Both microbes require no organic energy source. Nitrosamonas obtains its energy by oxidizing ammonia to nitrite, and nitrobacter by oxidizing the nitrite to nitrate. Therefore, the nutrient effects of inorganic fertilizers on nitrosomonas and nitrobacter are largely limited to the ammonia that they supply directly as ammonium salts, or indirectly in urea or organic materials.

In the present study, urea was used as the source of nitrate for the growing vegetables. The remarkable effect of urea on the accumulation of nitrate in the vegetables is demonstrated in Fig. 1. All five species of vegetables studied, gave bimodal patterns of nitrate accumulation. This phenomenon may be attributed to the two stages of urea supplement. During the course of cultivation, 2·8 kg urea-N/are was broadcast prior to the planting and an additional total of 2·7 kg urea-N/are was side-dressed 5, 9 and 15 days later. Similar results have been described by several authors. Berker, Peck & MacDonald (1971) showed that urea, ammonium nitrate, and potassium nitrate side-dressed onto a rapidly growing spinach crop increased the nitrate concentration in its leaves. Peck, Barker, MacDonald & Shallenberger (1971) observed that nitrate accumulation from side-dressed urea or potassium nitrate tended to increase in the plant with time after application.

Further evidence that most vegetable nitrate comes from the soil has come from our soil analyses. Soil samples from the virgin area that had never grown any vegetable contained 20–38 ppm nitrate, while the soils in which field mustard, broad-leaf mustard and long-footed cabbage had been grown contained 2·2, 3·3 and 3·8 ppm nitrate, respectively. This indicated that most nitrate produced in the soils had been accumulated by the growing vegetables.

Nitrate in vegetables is derived primarily from nitrate added to or formed in the soil, therefore, the N supply is the most significant factor governing nitrate accumulation in vegetables (Brown & Smith, 1966).

Factors affecting the level of nitrite in vegetables

Although the nitrate content in fresh vegetables is quite high (Fig. 1), the nitrite content is relatively low (Fig. 2). Nitrate may be converted to nitrite during storage of plant products as a result of bacterial action or plant nitrate reductase activity. Both nitrate reductase and nitrite reductase are able to affect the final nitrite-N concentration in the growing vegetables. In the present study, the activities of these two enzymes were found to be comparable to each other (Fig. 3 & 4). This may be the reason why nitrite-N did not accumulate in the growing vegetables (Fig. 2).

Storage temperature can affect the level of nitrite-N in vegetables (Table 3). At −10, 2 and 15°C, the samples showed practically no change in nitrite-N level, but at 26°C, the level was increased as much as 10- and 600-fold after 2 and 3 days, respectively. These results strongly suggest that parasitic microorganisms may play a crucial role in the changes of nitrite-N levels in the vegetable samples. The association between bacterial multiplication and the production of nitrites in carrot juice has been demonstrated by Hicks, Stall & Hall (1975). Microorganisms may have been present on the surface of the vegetables and/or within the root. Healthy plant tissues have been found to contain variable levels of saprophytic bacteria (Samish & Etinger-Tulcznoka, 1963). Such bacteria can multiply resulting in much higher levels within the tissue when conditions such as temperature and moisture are particularly favourable for bacterial growth.

From the studies, there does not appear to be any basis for concern about the endogenous nitrite content of fresh vegetables such as short-petioled cabbage, long-footed cabbage, field mustard, broad-leaf mustard and water convolvulus. Vegetables that are to be stored should be cooled promptly and held at a low temperature.

The problem of nitrate in food

Concern has been repeatedly expressed in the scientific and popular literature about the extent and effects of nitrate and nitrite in our diet. In order to provide some perspective on the dietary levels of these compounds, a series of studies has been carried out in this laboratory to evaluate the possible contribution of various sources of nitrate and nitrite to the Chinese diet (Lin, 1978; Lin & Lue, 1979). Of 57 kinds of vegetables analysed, six vegetables contained 200 ppm or more nitrite, and eight vegetables contained 500 ppm or more nitrate (Lin & Lue, 1979). Among 186 samples of sausage (a Chinese recipe of cured meat) purchased from different local markets, 18 contained 200 ppm or more nitrite, and 62 contained 500 ppm or more of nitrate (Lin, 1978). Vegetables are the most popular food of Chinese people. It is therefore estimated that most of the nitrate intake of the average Chinese inhabitant comes from vegetables and cured meat. A similar estimate was made for the average US citizen (White, 1975).

The effect of dietary nitrate on the formation of salivary nitrite has been studied (Tannenbaum *et al.* 1976), and the possible association of this nitrite with the endogenous formation of carcinogenic *N*-nitroso compounds has also been considered (Spiegelhalder *et al.* 1976; Tannenbaum *et al.* 1974; Walters, Carr, Dyke, Saxby & Smith, 1979).

The presence of nitrite and secondary amines together leads to the formation of nitrosamines, which are mutagenic and carcinogenic compounds (Committee on Nitrate Accumulation, 1972). Nitrite may be formed from the nitrate in vegetables or may be added to food as a preservative; secondary amines are present from a number of sources including fish, shrimps (Lin & Lai, 1980), tobacco smoke and flavourings (Lijinsky & Epstein, 1970). It is likely that the nitrate in vegetables may be involved in the endogenous formation of carcinogenic *N*-nitroso compounds but more data are needed to confirm this. White (1975) estimated that four-fifths of the nitrate intake is from vegetables, therefore, control of the nitrate content in vegetables may be a feasible way to control the endogenous formation of *N*-nitroso compounds. Accordingly, much more effort should be made to develop new methods of cultivating vegetables with reasonably low nitrate contents.

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MUTAGENICITY TESTING OF COFFEE: A STUDY OF PROBLEMS ENCOUNTERED WITH THE AMES SALMONELLA TEST SYSTEM

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Abstract—Instant coffee and fresh-brew coffee gave comparable results when tested in the Ames Salmonella mutagenicity test system. At maximal concentrations of 25–30 mg/plate, both coffees induced an effect only in the *Salmonella typhimurium* strain TA100 without S-9 mix, and this effect was only weak (a doubling of the spontaneous mutation rate). The effect was completely abolished in the presence of the microsomal fraction, S-9 mix, and further studies provided evidence that metabolic deactivation was the mechanism involved. Several factors of possible relevance to the findings, such as caffeine level, histidine content, autoclaving and pH, were investigated, but none significantly influenced the results obtained with the Salmonella test system. However, the bactericidal effects and 'mutagenicity' of the coffees were closely related and a 'false-positive' effect due to bactericidal interaction cannot be totally excluded.

INTRODUCTION

It has recently been suggested that heated foods, including coffees, contain mutagens that are active in the Ames test (Nagao, Takahashi, Yamanaka & Sugimura, 1979; Sugimura, 1978). It has also been postulated that a correlation exists between mutagenicity and carcinogenicity (McCann, Choi, Yamasaki & Ames, 1975). However, such a correlation has been contested (Ashby & Styles, 1978; Rinkus & Legator, 1977) and the actual carcinogenicity of heated food products, as tested in lifespan tests in experimental animals, remains uncertain.

Coffee has been found to induce a very weak mutagenic effect in the Ames test but this did not occur in the presence of a microsomal fraction (Aeschbacher & Würzner, 1980; Nagao *et al.* 1979). Since the complexity of a substance such as coffee, which contains several hundred components (Oser, 1978), could lead to artefacts in the Ames test, several factors that may interfere with the results were investigated in the present study.

EXPERIMENTAL

Test organisms and procedure. The standard Ames test for mutagenicity was used throughout the study and the regularly controlled *Salmonella typhimurium* tester strains TA1535, 1537, 1538, 98 and 100 were used. These were kindly supplied by Dr. B. N. Ames, University of California, Berkeley, CA, USA. The more detailed investigations were carried out with the most sensitive strains, TA98 and TA100 and instant coffee. For revertant counts, about 10^8–10^9 bacteria/ml were applied to minimal agar plates, whereas for total bacterial counts a 10^5 dilution of the 10^8–10^9/ml suspension was used on complete agar plates. Each plate was inoculated with 0·1 ml of an overnight nutrient broth culture, and 0·5 ml of coffee solution or of a control solution (see below) was added. For each concentration of test compound or control, three complete agar plates and four minimal agar plates were used. The media and soft agar were prepared as described by Ames, McCann & Yamasaki (1975), using a sterile semi-automated system, Agarmatic, and a Technomat distributing system. Petri plates were incubated for 3 days at 37°C and colonies were counted with an automated colony counter, Biotran II. Regular repetition of the assays permitted checks for variations of revertants in the Ames test. As criteria for a mutagenic effect the commonly suggested doubling of the spontaneous rate was used (Ames *et al.* 1975). In all assays in which an increased number of revertants was observed, replication techniques were used to avoid misinterpretations.

Metabolizing system. The microsomal fraction (S-9) was obtained from Sprague–Dawley rats (supplied by Charles River, Saint-Aubin-les-Elbeuf, France), which were given a single ip injection of 500 mg Aroclor 1254/kg and killed 5 days later. Unless otherwise indicated, 1 ml S-9 mix contained 0·1 ml of the 9000-g supernatant prepared from fresh rat liver. Cofactor solution was prepared according to Ames *et al.* (1975). To check whether deactivation of test samples was due to metabolism or to protein binding, 0·5 ml of either calf serum or cofactor-free S-9 mix (0·9 ml phosphate buffer/ml S-9 mix) was added per plate. The microsomal-enzyme activity of the 9000-g supernatant used for metabolic activation was regularly checked by measuring ethylmorphine *N*-demethylase activity (Dalton & Di Salvo, 1972).

Positive controls. 2-Nitro-1,4-phenylenediamine was used to check the inducibility of the tester strains, and aflatoxin B_1, benzo[a]pyrene and 2-aminoanthracene were used to check the metabolic activity of the system.

Preparation of coffee samples. Instant and freshly brewed coffees were always diluted in hot tap-water. Unless otherwise indicated in the tables, the solutions were then sterilized for 20 min in an autoclave at 120°C for the plate tests. The other coffee solutions were sterilized by filtering through a Millipore (0·45 μm) filter (Millipore AG, Kloten). Fresh-brew coffee was prepared on a Melitta-filter from the the same mix-

ture of roasted coffee beans as was used for instant coffee, in amounts to produce coffee of a similar concentration as a cup of coffee normally prepared at home (50 g coffee/litre water). The coffee so obtained was then freeze-dried and rediluted in the same way as the instant coffees. For the instant coffee tests, commercially available instant coffee was used. Pure caffeine, obtained from Fluka AG, Buchs, was added to samples of a decaffeinated instant coffee to investigate the possibility of a synergistic effect between caffeine and other coffee constituents. The coffees used for all the other tests contained caffeine.

Determination of histidine in coffee. For the determination of total amino acids, instant coffee was refluxed with 6 N-HCl at 110°C for 22 hr. To determine free amino acids, instant coffee was treated with potassium citrate buffer (pH 2) for 2 hr followed by filtration through a Millipore NAWPO 1300 filter (Millipore AG). Both preparations were analysed on a Technicon Amino Acid Analyzer (Spackman, Stein & Moore, 1958). The possible effect of relevant levels of histidine on the results of the Ames test was studied.

RESULTS

Assays without S-9 mix

Both fresh-brew ('home-brew') and instant coffee at doses up to 25–35 mg/plate increased the number of spontaneous revertants in *S. typhimurium* TA100 about 2 to 2·5 times in the Ames (direct plating) tests (Tables 1 and 2). Both coffees increased the spontaneous rate to a similar extent and were also bactericidal (see below). Fresh-brew coffee did not increase the number of revertants in TA98, the other strain in which it was tested (Table 3). Neither was any such increase demonstrated when instant coffee was tested with TA98 or with the other normally used Salmonella tester strains TA1535, 1537 and 1538 (Table 4).

Metabolic deactivation

Addition of the microsomal preparation, S-9 mix, to the assay plates together with either fresh brew or instant coffee abolished the effect observed with strain TA100 in the absence of S-9 mix, no significant increase over the spontaneous (0 mg/plate) reversion level being observed (Tables 1 and 2). Tested together with S-9 mix, neither coffee caused an increase over the spontaneous reversion levels of the other tester strains (Tables 3 and 4).

To discover whether the inhibition of the effect seen in TA100 was due to metabolic deactivation by the S-9 mix or to the binding of active compounds to protein, further studies were undertaken with instant coffee using calf serum and S-9 mix without cofactors. In a series of tests on all five Salmonella strains, S-9 mix without cofactors was found to have the same detoxifying effect on the instant coffee as did S-9 mix with cofactors (Tables 2 and 4). Strains TA98 and TA100 were then used to compare the results obtained with S-9 mix and cofactors with those using serum protein. It was apparent that serum protein did not have the deactivating effect of the S-9 mix, since the results of the TA100 assay incorporating serum protein were similar to those obtained with this strain in the absence of S-9 mix (Table 2). No significant differences were observed between the results obtained with and without S-9 mix and with serum

Table 1. *Results of Ames tests on sterilized and unsterilized 'home-brew' coffee, using* S. typhimurium *TA 100*

Concn of coffee (mg/plate)	Assay no...	Counts/plate							
		Without S-9 mix				With S-9 mix			
		No. of revertants		Total count		No. of revertants		Total Count	
		1	2	1	2	1	2	1	2
Autoclaved sample									
0		96	87	757	544	100	88	663	471
5		130	122	674	370	135	117	791	507
15		159	156	123	0	142	117	792	457
25		210	212	0	0	145	118	788	517
35		218	31	0	0	148	119	796	558
50		80	0	0	0	166	143	751	496
75		0	—	0	—	166	—	676	—
Filtered sample									
0		114		343		135		342	
5		124		282		129		448	
15		169		0		127		476	
25		101		0		154		475	
35		0		0		146		494	
50		0		0		159		501	
75		—		—		—		—	
Unsterilized sample									
0		98		607		97		588	
5		125		77		122		678	
15		144		0		127		731	
25		203		0		132		734	
35		0		0		151		719	

Table 2. *Effects of S-9 mix, with and without cofactors, and of serum protein on the results of Ames tests on autoclaved instant coffee using* S. typhimurium *TA100*

Concn of coffee (mg/plates)	Assay no...	Counts/plate															
		Without S-9 mix				With S-9 mix				With S-9 mix—without cofactors				With serum protein			
		No. of revertants		Total count		No. of revertants		Total count		No. of revertants		Total count		No. of revertants		Total count	
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Metabolic deactivation study																	
0		129	176	207	177	159	178	195	181	189	203	258	198				
5		132	185	194	153	167	182	261	168	163	192	248	196				
15		161	202	0	37	156	182	253	178	158	188	260	177				
25		249	336	1	2	188	174	234	179	216	214	267	195				
35		355	0	0	0	160	186	—	181	185	200	254	185				
Protein-binding study																	
0		118	90	655	640	145	84	638	598					116	86	668	674
5		139	109	605	573	156	108	672	629					145	123	574	534
15		167	125	0	43	159	106	577	553					226	120	21	308
25		219	160	0	0	166	129	572	569					0	162	0	59
35		0	0	0	0	164	0	665	572					0	152	0	1

Table 3. *Results of Ames tests on autoclaved and filtered 'home-brew' coffee in* S. typhimurium *TA98*

Concn of coffee (mg/plate)	Assay no...	Counts/plate						
		Without S-9 mix				With S-9 mix		
		No. of revertants		Total count		No. of revertants		Total count
		1	2	1	2	1	2	1
		Autoclaved sample						
0		20	22	1036	573	27	31	1463
5		32	17	27	5	27	21	1490
15		24	15	6	0	20	16	1374
25		21	14	0	0	26	19	1292
35		14	10	0	0	24	21	1324
50		15	0	0	0	24	18	1323
75		0	—	0	—	25	—	1331
		Filtered sample						
0		15		538		21		1060
5		21		10		20		1162
15		19		0		18		1357
25		11		0		17		1307
35		0		0		21		1289
50		0		0		17		1339

protein in tests of instant coffee using TA98 (Table 5). In order to establish whether a variation in the amount of S-9 fraction in the S-9 mix would affect the results, 0·3 ml S-9/ml mix was used instead of the usual 0·1 ml S-9, but similar results were obtained with both S-9 concentrations (Table 6).

Synergism study on caffeine

The possible potentiating effect of caffeine on other compounds present in coffee was investigated in strain TA100. It appeared that there was no such effect, since decaffeinated instant coffee with or without added caffeine gave results similar to those obtained with standard instant coffee (Table 7).

Influence of histidine on the Ames test

The influence of histidine present in instant coffee was also studied. Biochemical analyses demonstrated no free histidine in instant coffee, but showed total bound histidine to be present at a level of 0·14 mg/100 mg. Application of 35 mg instant coffee to a plate produces a total content of 70 μg histidine/plate (49 μg in coffee and 21 μg free histidine added with the soft agar). This corresponds to a histidine concentration of about 1·7 mM, compared with 0·5 mM for the histidine solution normally used in the Ames test (Ames *et al.* 1975). Free histidine at such a level does increase the number of revertants in TA100; the number of spontaneous revertants was 160, 182, 198, 222, 253, 281 and 285 with histidine concentrations of 0·5, 0·73, 1·18, 1·64, 2·09, 2·55 and 3·00 mM respectively.

Effect of sample preparation

Problems due to the method of sample preparation were also investigated. Specifically the effects of sterilizing the coffees either by filtration through a Millipore filter (0·45 μm) or by autoclaving at 120°C for 20 min were compared, since it was recognized that autoclaving might form new compounds or destroy active constituents, while filtration could retain some of the coffee constituents. Similar results were obtained with the two methods of sterilization in several assays of home-brew coffee with strains TA100 and TA98, although the filtered coffee showed some tendency towards a lower activity (Tables 1 and 3). These results suggest that the mode of sterilization does not influence the results, especially since similar results were obtained with unsterilized fresh-brew coffee and strain TA100 (Table 1). This conclusion was supported by the results obtained with instant coffee.

Effect of pH

The pH of the coffee solutions used increased from about 5·0 to 5·5 with increasing coffee concentrations. In this range the pH does not interfere with the Ames test; in a separate assay using McIlvaines buffer only a pH below 3·5 showed an effect.

Bactericidal effect of coffee

Whereas all the above-mentioned results were relevant to the study of a possible mutagenic effect (as reflected in an induction of reversion), the total surviving bacteria (total counts) served as a measure of the bactericidal effect of the coffees tested. The total counts, however, allow only a rough estimate and their direct comparison with the number of revertants is not feasible, since for the total counts a much greater bacterial dilution was used and the bacteria were plated on complete agar rather than on minimal agar. It was found (Table 8) that the bactericidal effect of instant coffee increased the more the bacterial suspension was diluted. Nevertheless, it is interesting to note that the microsomal fraction (S-9 mix) not only abolished the weak mutagenic effect demonstrated in strain TA100 in its absence, but also the bactericidal effect that home-brew and instant coffees exerted in all incubations without S-9 mix (Tables 1–5). In gen-

Table 4. *Results of Ames tests on autoclaved instant coffee in several strains of* S. typhimurium

Concn of coffee (mg/plate)	Assay no...	Counts/plate											
		Without S-9 mix				With S-9 mix				With S-9 mix—without cofactors			
		No. of revertants		Total Count		No. of revertants		Total count		No. of revertants		Total count	
		1	2	1	2	1	2	1	2	1	2	1	2
						TA98							
0		21	25	200	699	20	24	313	723	36	37	299	764
5		30	33	144	303	19	27	272	731	36	36	315	679
15		29	51	20	32	25	29	313	719	29	31	298	733
25		8	40	1	0	25	33	323	627	0	32	298	596
35		4	26	0	0	26	32	387	733	0	33	320	651
						TA1535							
0		28		232		38		232		28		247	
5		36		232		30		246		29		262	
15		41		40		28		229		27		231	
25		43		0		20		335		20		242	
35		0		0		27		—		20		272	
						TA1537							
0		12	7	713	230	14	10	758	349	10	7	773	317
5		24	8	184	234	9	9	763	379	14	9	763	361
15		20	10	7	48	9	9	760	372	11	7	721	372
25		13	8	0	0	10	9	736	378	14	10	759	347
35		11	1	0	0	15	9	747	354	13	8	723	319
						TA1538							
0		13	12	179	68	11	12	194	292	22	20	184	340
5		15	8	20	17	9	8	186	299	20	18	198	305
15		19	11	1	0	15	9	183	321	17	14	173	296
25		15	9	1	0	16	7	193	304	18	11	187	293
35		4	0	0	0	18	13	187	302	21	11	213	322

Table 5. *Effects of serum protein on the results of Ames tests on autoclaved instant coffee using* S. typhimurium *TA98*

Concn of coffee (mg/plate)	Assay no...	Counts/plate																	
		Without S-9 mix						With S-9 mix						With serum protein					
		No. of revertants			Total count			No. of revertants			Total count			No. of revertants			Total count		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0		15	18	11	1140	1020	511	27	32	23	1104	1134	571	17	24	14	1061	491	491
5		—	52	16	—	211	491	—	50	24	—	1112	498	—	43	13	—	109	102
15		51	45	16	2	0	21	46	50	24	891	963	46	35	44	16	17	0	6
25		31	39	16	0	0	0	32	47	27	797	635	177	42	24	19	0	0	0
35		25	22	0	0	0	0	39	48	27	857	1040	325	32	0	0	0	0	0
50		0	—	—	0	—	—	38	—	—	543	—	—	0	—	—	0	—	—
75		3	—	—	0	—	—	31	—	—	965	—	—	0	—	—	0	—	—

Table 6. *Effect of an increased concentration of the microsomal fraction on the Ames assay of filtered instant coffee using* S. typhimurium *TA100*

Concn of coffee (mg/plate)	Counts/plate			
	Standard S-9 concn		High S-9 concn*	
	No. of revertants	Total count	No. of revertants	Total count
0	156	367	215	350
5	161	422	215	450
15	150	472	202	440
25	153	489	218	420
35	142	492	250	350
50	157	497	221	350
75	163	479	233	320

*The 'high concentration' preparation contained 0·3 ml S-9 fraction/ml cofactor solution, compared with the standard concentration of 0·1 ml/ml.

eral, the coffees had a bactericidal effect on auxotrophs already present in low concentrations (5–15 mg/plate), whereas it only became bactericidal to revertants (prototrophs) at concentrations above 35 mg/plate.

DISCUSSION

Comparison of the activity of two kinds of coffee, instant and regular, in the Ames test revealed that both had the same properties. A borderline effect was caused only by exceedingly high doses of either coffee, as may be seen by comparing the results with the recommendations of Ames *et al.* (1975) or de Serres & Shelby (1979). This effect was observed, moreover, only in the especially sensitive Salmonella tester strain TA100, which detects base-pair substitutions, and not in the less sensitive strain TA1535 which also detects base-pair substitutions. The coffees did not induce frame-shift mutations in the sensitive tester strain TA98 nor in the less sensitive strains TA 1537 and TA1538.

The results of Nagao *et al.* (1979), who found that regular (brewed) coffee and instant coffee both induced a weak effect in Salmonella TA100, were thus confirmed, despite the fact that they used a modified Ames test with pre-incubation and dissolved the coffee in dimethylsulphoxide, whereas we used the well-established Ames test (Ames *et al.* 1975) and diluted the coffees in water.

The microsomal activating system did not convert any substances present in coffee into compounds mutagenic to the tester strains used. On the contrary, the microsomal preparation, S-9 mix, abolished the doubling of revertants observed when the coffees were tested in strain TA100 without a metabolizing system, a further confirmation of the findings of Nagao *et al.* (1979). Bactericidal effects and the increase in revertants caused by the coffees seemed to be related, since addition of S-9 mix also abolished the bactericidal effect both for revertants and for total surviving bacteria (auxotrophs).

To judge the implication of this deactivation capacity in terms of risk assessment for mammals, it was necessary to investigate whether this deactivation was due to metabolism or to other factors, such as protein binding. A comparison was therefore made of the results of coffee tests carried out in the presence of S-9 mix with cofactors, S-9 mix without the cofactors or calf-serum protein. It was observed that the S-9 mix had the same deactivating effect on coffee whether or not cofactors were present, whereas the calf serum did not have this property. This leads to the conclusion that the S-9 mix does not require additional NADPH-generating systems for deactivation, in agreement with the findings of Hope (1979). Hence it

Table 7. *Investigation in* S. typhimurium *TA100 (without S-9 mix) of a possible synergistic effect between caffeine and other coffee components*

Sample	Concn of coffee (mg/plate)	Addition (+) of caffeine*	Revertants (no./plate)
Control	0	—	138
		+	160
Instant coffee	25	—	213
(standard)	50	—	246
Instant coffee			
(decaffeinated)	25	—	228
		+	205
	50	—	277
		+	283

*Caffeine was added to the decaffeinated coffee in an amount to produce the concentration (4%) normally present in coffee.

Table 8. *Relation between the survival of* S. typhimurium *TA100 in the presence of different concentrations of instant coffee and the concentration of the bacterial suspension*

Concn of coffee (mg/plate)	Total no.* of surviving bacteria (auxotrophs)/plate with suspension dilutions† of		
	10^0	10^{-4}	10^{-6}
0	+ + +	+ + +	126
5	+ + +	+ + +	89
10	+ + +	371	9
15	+ + +	97	—
20	+ + +	11	—
25	+ + +	—	—
30	+ + +	—	—
35	+ + +	—	—
40	+ + +	—	—
45	+ + +	—	—
50	+ + +	—	—

*By + + + is indicated a large number of colonies, which could not be counted.
†Strain TA100 was plated out on complete agar either undiluted or diluted 10^{-4} or 10^{-6} times and exposed to a range of instant-coffee concentrations.

is most likely that the observed property of the microsomal preparation is due to metabolic deactivation rather than to other factors.

This observation is important, since De Flora (1978) showed that compounds that undergo such metabolic deactivation in the Ames test are in general not carcinogenic in mammals. It has also been proposed that man has evolved with enzymes that can inactivate small amounts of mutagens present in food quickly enough to avoid any risk (Sugimura, 1978).

The complexity of the problems that occur when complex substances such as coffee are tested in the Ames test led us to further investigations. In fact caffeine, which is known to enhance the effect of some mutagens (Kihlman, 1977), did not potentiate the effect of other compounds present in coffee. It seemed also that preparing the coffees for testing did not modify them to a degree that influenced the results of the Ames test.

There remained the possibility that the histidine present in coffee would interfere with the Ames test. Enough bound histidine was present in the coffee to increase the number of revertants. Further investigation revealed, however, that the Salmonella could not use the histidine when it was bound to large molecules. Also a deactivation of coffee by the microsomal fraction would not be possible if the increase of revertants in TA100 were due to histidine. Hence, the histidine present in coffee was not responsible for the observed increase in revertants.

It should be borne in mind that the Ames test is a screening test and cannot take into account various factors occurring in mammals (Ashby & Styles, 1978; Bartsch, 1976). In such cases chronic mammalian tests are necessary. A 2-yr feeding study (Würzner, Lindström, Vuataz & Luginbühl, 1977) indicated that the tumour incidence in several groups of coffee-treated rats was no higher than that in the controls.

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THE OCCURRENCE OF OCHRATOXIN A IN MOULDY BREAD AND FLOUR

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Abstract—Fifty samples of mouldy bread and seven samples of mouldy flour lumps were analysed for mycotoxins by thin-layer chromatography (TLC). Ochratoxin A was detected in one sample of bread at a level of 0·21 mg/kg and in two of the samples of mouldy flour lumps at levels of 2·9 and 0·49 mg/kg. The presence of ochratoxin A was confirmed by high-performance liquid chromatography (HPLC). Citrinin was also detected in one of these samples of mouldy flour lumps, but was present at levels too low to be quantitated. TLC indicated the presence of patulin in two of the samples of bread, but subsequent HPLC did not confirm this finding.

INTRODUCTION

Although aflatoxin B_1, aflatoxin G_1, patulin, ochratoxin A, citrinin and sterigmatocystin have been produced by moulds experimentally grown on bread (Reiss, 1975, 1976 & 1977) only aflatoxins (Frank & Eyrich, 1968; Spicher, 1970; Hansen & Jung, 1973) and patulin (Tyllinen, Raevuori, Karppanen & Garry-Andersson, 1977) have been reported to have been detected on samples of spontaneously mouldy bread and these have usually been 'mixed' German bread (wheat/rye). The aflatoxin was found to have penetrated 7 cm into the crumb of an uncut mouldy wholemeal loaf (Hansen & Jung, 1973).

In a survey of home-stored foods three out of 15 unspecified bakery products yielded aflatoxin-producing isolates (Torrey & Marth, 1977) but other workers have failed to isolate toxigenic moulds from samples of mouldy bakery products involving consumer complaints, including one allegedly linked with illness (van Walbeck, Scott & Thatcher, 1968). Seventy different moulds were isolated from wheat flour and bread in the USA and among them were some toxigenic Penicillia including *P. cyclopium* and *P. citrinum*. It was considered that in the case of bread the mould contamination must have occurred after baking since the moulds present in the flour would have been destroyed at the baking temperature (Bullerman & Hartung, 1973). In the UK there has been only one reported incidence of mycotoxin-contaminated mouldy flour. In this case ochratoxin A (up to 6·25 mg/kg) was produced by *P. cyclopium* Westling (Richardson, Flude, Patterson, Mackenzie & Wakefield, 1978).

Ochratoxin A is a mycotoxin produced by a variety of moulds in the genera Aspergillus and Penicillium but the latter are able to produce it at lower temperatures and therefore are more likely to be implicated as toxigenic species in countries with temperate climates (World Health Organization, 1979). The toxin has been shown to cause a kidney disease in pigs known as porcine nephropathy and there is some evidence correlating levels of foodborne ochratoxin A with a similar human disease (Balkan endemic nephropathy) which only occurs in certain areas of the Balkan peninsula. However, there is no proof that ochratoxin A is causally involved in human disease (World Health Organization, 1979). More recent work with rats has cast doubt on the role of ochratoxin A in the etiology of nephropathy and results suggest that the toxin exerts a specific chronic action on the proximal renal tubule which is more consistent with tubular necrosis (Berndt & Hayes, 1979).

Waste flour and bread are commonly used as components of pig feed and since bread in particular has a tendency to become mouldy because of its high moisture content, the possible occurrence of mycotoxins in mouldy flour and bread was investigated.

EXPERIMENTAL

Samples. Thirteen samples of different types of bread that had become mouldy in domestic environments were obtained, together with a further six samples from retail sources and 31 samples (15 sliced white loaves, 12 sliced brown loaves and four wholemeal loaves) from commercial bakeries geographically distributed around England. Seven samples of mouldy flour lumps sieved from bulk silo flour at bakeries were also obtained.

Analytical procedure. The samples of flour lumps were ground in a pestle and mortar to a homogeneous powder and portions of the bread samples were selected randomly for analysis.

All of the samples were extracted, cleaned-up and screened for 11 mycotoxins (aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A, zearalenone, sterigmatocystin, T-2 toxin, diacetoxyscirpenol, citrinin and patulin) by thin-layer chromatography (TLC) according to a published procedure (Roberts & Patterson, 1975), slightly modified. The modification involved the use of Whatman LK5D TLC (Whatman Inc., Maidstone, Kent)

plates which permitted the application of 30 μl aliquots of sample extract for greater sensitivity without overloading the plate.

High performance liquid chromatography (HPLC) was used for the confirmation and quantitation of patulin and ochratoxin A. The apparatus used and the chromatographic conditions for the analysis of ochratoxin A were as previously described (Osborne, 1979). The spot on the TLC plate suspected to represent ochratoxin A was scraped off, the silica gel was extracted with acetonitrile and the extract was analysed by HPLC. HPLC analysis was also carried out on an extract that had been prepared by the method of Roberts & Patterson (1975) and further cleaned up by back-extraction with sodium bicarbonate solution followed by acidification of the separated aqueous phase and re-extraction into chloroform after acidification. The chloroform was evaporated off and the residue was taken up into acetonitrile for chromatography. For the analysis of patulin a Hypersil 5 ODS column (Anachem Ltd., Luton, Beds.) was used and the mobile solvent was acetonitrile–water (87·5:12·5, v/v) pumped at a flow rate of 0·5 ml/min. The chloroform extract obtained by the procedure of Roberts & Patterson (1975) was evaporated to dryness and taken up in acetonitrile for chromatography. Patulin was detected by ultraviolet absorption at 280 nm.

RESULTS

Ochratoxin A was detected in one sample of bread from a domestic environment at a level of 0·21 mg/kg and in two of the samples of mouldy flour lumps at levels of 2·9 and 0·49 mg/kg. Citrinin was also detected in one of these samples of mouldy flour lumps but at levels that were too low to be quantitated. There was a TLC spot corresponding with patulin in chromatograms of extracts of two separate mouldy bread samples from domestic environments but the presence of patulin in these extracts was not confirmed by HPLC. No other mycotoxins were detected in any of the samples.

The presence of citrinin was confirmed by a change in the colour of its fluorescence when the TLC plate was sprayed with 20% ethanolic aluminium chloride solution and heated at 100°C for 5 min, by two-dimensional TLC (Patterson & Roberts, 1979) and by co-chromatography in two different solvent systems using plates impregnated with oxalic acid (Gorst-Allman & Steyn, 1979).

Ochratoxin A was confirmed by a change in the colour of its fluorescence when the plate was exposed to ammonia fumes or sprayed with aluminium chloride as above (Roberts & Patterson, 1975).

Ochratoxin A was further confirmed by two-dimensional TLC (Patterson & Roberts, 1979) and by HPLC. HPLC analysis of the extract of the suspect spot on the TLC plate revealed a peak identical to that of authentic ochratoxin A, and the presence of ochratoxin A in the extract prepared by the method of Roberts & Patterson (1975) and further cleaned-up by the method of Osborne (1979) was also confirmed by HPLC.

DISCUSSION

It is apparent that rigorous confirmation of suspected mycotoxins must be carried out before conclusions may be drawn. It is significant that TLC analysis alone indicated patulin contamination of bread in two cases but subsequent HPLC analysis failed to confirm this finding. Patulin would not be expected to be found in mouldy bread or flour because of its instability in the presence of the sulphydryl-containing amino acids of cereal proteins (Reiss, 1977; Scott & Somers, 1968). These two observations cast doubt on the previous report of the occurrence of patulin in mouldy bread (Tyllinen *et al.* 1977).

There have been three previous reports of the occurrence of aflatoxins in mouldy bread (Frank & Eyrich, 1968; Hansen & Jung, 1973; Spicher, 1970), all in Germany where bread is usually made from rye as well as wheat. No aflatoxins at all have been found at low limits of detection (0·3 μg/kg) in the present study of mouldy English bread and flours.

The presence of citrinin as well as ochratoxin A in one sample of mouldy flour is not surprising since they are both produced by Penicillia which have previously been found in the mycoflora of flour (Bullerman & Hartung, 1973). The presence of ochratoxin A at low levels in only one out of 50 samples of mouldy bread need give no cause for alarm. While the finding of ochratoxin A in two out of seven samples of mouldy flour is interesting and indicates the need for the study of further samples, its presence at the levels found, which were lower than those reported previously (Richardson *et al.* 1978), cannot be considered to present any risk to human health.

In conclusion therefore, the practice of feeding stale bread, which may be mouldy, to pigs should not be condemned on the grounds of possible contamination of the meat or kidneys destined for human consumption.

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PATHOLOGICAL CHANGES IN RATS FED THE CRAMBE MEAL-GLUCOSINOLATE HYDROLYTIC PRODUCTS, 2*S*-1-CYANO-2-HYDROXY-3,4-EPITHIOBUTANES (*ERYTHRO* AND *THREO*) FOR 90 DAYS

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Abstract—2*S*-1-Cyano-2-hydroxy-3,4-epithiobutanes (*erythro* and *threo*) isolated from the seed of *Crambe abyssinica*, were fed to groups of six weanling rats at levels of 0, 75, 150 or 300 ppm in the diet for 90 days. The higher dose groups showed poor growth and increased serum levels of alkaline phosphatase, total bilirubin, glutamic–oxaloacetic transaminase, glutamic–pyruvic transaminase and ornithine carbamyl transferase. The severity and occurrence of renal and hepatic lesions were dose-dependent. Renal alterations consisted of hypertrophy of proximal tubular epithelial cells with prominent karyomegaly. Hepatic lesions consisted primarily of megalocytosis of the hepatocytes and bile-duct hyperplasia with disruption of the normal hepatic architecture. Half of the rats in the high-dose group had karyomegaly of pancreatic acinar cells.

INTRODUCTION

Cruciferous plants, which are commonly cultivated for use as vegetables, condiments, oilseeds and forage, contain glucosinolates (GS) that are enzymatically hydrolysed to a number of biologically active nitriles or isothiocyanates. It has been shown that nitrile formation is more likely to occur during autolysis of the crushed, wet, natural plant material (VanEtten & Daxenbichler, 1971; VanEtten, Daxenbichler, Peters & Tookey, 1966; VanEtten & Tookey, 1979). Epiprogoitrin (*S*-2-hydroxy-3-butenyl-GS), the major GS of crambe seed, may by hydrolysed in defatted meal by endogenous enzymes to yield goitrin (*R*-5-vinyloxazolidine-2-thione) which supresses thyroidal iodine uptake and causes thyroid hyperplasia and hypertrophy (Greer, 1962). Alternatively, during autolysis of crushed, recently harvested seed below 45°C, the hydrolysis of epiprogoitrin preferentially follows a pathway that results in the formation of three nitriles, *S*-1-cyano-2-hydroxy-3-butene and two diastereomeric 2*S*-1-cyano-2-hydroxy-3,4-epithiobutanes (CHEB; Daxenbichler, VanEtten & Wolff, 1968). The conditions for the formation of these substances have been defined and the biological effects of the autolysed meal and of the nitrile mixture isolated from autolysed meal have been briefly described (VanEtten, Gagne, Robbins, Booth, Daxenbichler & Wolff, 1969). This study was undertaken to determine the pathological effects of feeding two of these purified nitriles, *erythro*- and *threo*-CHEB at various concentrations to young rats for 90 days.

EXPERIMENTAL

Seed of *Crambe abyssinica* Hochst ex R. E. Fries was stored at 5°C until it was used. The seed was dehulled, flaked, and defatted by percolation with pentane–hexane (b.p. 33–57°C) at room temperature. The preparation of a typical batch of CHEB was as follows. A total volume of 400 ml water was added, in 50–100-ml aliquots, to 250 g defatted seed meal. After each addition, the mixture was quickly blended with a large spatula. After all of the water had been added, the wet meal was allowed to autolyse for a further 20 min. Sodium chloride (200 g) was blended into the slurry, followed by 3 litres dichloromethane. The resultant mixture was intermittently stirred by hand for 15 min and then the solids were removed by filtration through paper with the aid of Celite filter-aid. The solids were then extracted twice with 2 litres dichloromethane. The combined dichloromethane extracts were concentrated to 250 ml under vacuum at 45°C. A 0·5-ml aliquot was analysed by gas-liquid chromatography (GLC) as previously described (Daxenbichler & VanEtten, 1977) and it was found that the total dichloromethane extract of 250 g seed meal typically contained 1·4 g *threo*-CHEB, 1·6 g *erythro*-CHEB, 0·08 g 1-cyano-2-hydroxy-3-butene and 0·0 g 5-vinyloxazolidine-2-thione.

The dichloromethane concentrate was reduced to a syrup under vacuum at 45°C, mixed with 100 ml warm water and filtered through glass wool. The aqueous filtrate was extracted with dichloromethane

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(4 × 250 ml). These combined extracts were again reduced to a syrup and dissolved in 40 ml water. After centrifugation at 1560 **g** for 15 min to remove traces of solid material, the aqueous solution was applied to a 5 × 100 cm column of Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) using water as the eluent. The sample was allowed to enter the Sephadex bed by gravity flow. Fractions were collected at 30-min intervals (flow rate 53 ml/hr). The fractions were qualitatively analysed by thin-layer chromatography (TLC) on Precoated Silica Gel 60 F-254 (EM Laboratories, Inc., Elmsford, NY) plates. Ether–hexane (3:1, v/v) was used as the developing solvent and charring with H_2SO_4-dichromate was used for detection. Fractions 1–23 made up the column void volume (610 ml). Fractions 51–59 contained 1-cyano-2-hydroxy-3-butene, fractions 63–70 contained *threo*-CHEB and fractions 72–81 contained *erythro*-CHEB. The appropriate fractions were combined and extracted four times with twice their volume of dichloromethane. After evaporation of the solvent, 44 parts *threo*-CHEB were combined with 56 parts *erythro*-CHEB for use in the rat feeding studies. The only significant impurity that could be detected in the CHEB preparation by TLC and GLC analysis was a minor amount (about 1%) of 1-cyano-2-hydroxy-3-butene. Because the CHEB mixture was unstable and tended to polymerize when neat, it was stored in acetone solution at 0°C until it was incorporated into the feed.

Weanling male Sprague–Dawley rats, obtained from Simonsen Laboratories, Gilroy, CA, were fed a basal diet with the following composition: corn meal, 50%; soyabean meal, 30%; casein, 7%; corn oil (Mazola), 5%; Bernhart and Tomarelli salt mixture (United States Biochemical Corp., Cleveland, OH), 4%; vitamin diet fortification mixture (ICN, Cleveland, OH), 2·2%; corn starch, 1·65%; DL-methionine (ICN), 0·15%. The rats were housed three to a cage and were given feed and water *ad lib.* The animal room was maintained at 73°F and 50% relative humidity with equal periods of light and darkness.

Groups of six rats were fed a diet containing 0 (control), 75, 150 or 300 mg CHEB/kg. To prepare the test diets, the CHEB, dissolved in reagent-grade acetone, was thoroughly mixed into the basal diet to give a premix containing 900 mg/kg. After drying, the premix was appropriately diluted with additional basal diet. The diets were prepared at approximately 1-month intervals, placed in feed containers with screw caps and stored at −10°F until needed. Feed containers in the animal cages were replaced weekly. A check of the stability of the CHEB in the diet showed 100% recovery for at least 1 month when the diet was stored at −10°F. At room temperature, the recovery was 65% after 1 wk. Thereafter, the rate of loss decreased and recovery was 59% after 2 wk.

The rats were observed daily. Weight and feed intake were determined at approximately 1-wk intervals. After 90 days the rats were killed under ether anaesthesia by exsanguination *via* the brachial artery, and using potassium oxalate-treated syringes blood samples were obtained from the axillary space. Determinations on blood included erythrocyte, leucocyte and platelet counts (Coulter Counter Model ZBI, Coulter Electronics, Hialeah, FL), packed cell volume, haemoglobin concentration, and differential leucocyte counts. Plasma levels of glucose, albumin, total protein, alkaline phosphatase, urea nitrogen, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, ornithine carbamyl transferase and total bilirubin were determined using a Technicon Auto-Analyzer-II (Technicon Corp., Tarrytown, NY). Plasma sodium and potassium were determined using a Klina Flame Spectrophotometer (Beckman Instruments, Fullerton, CA). Analysis was carried out on samples of urine collected in the last week of the test. Occult blood, ketones, glucose, protein, bilirubin, urobilinogen and pH were estimated using Multistix (Ames Company, Elkhart, IN). Colour, specific gravity and the presence of urinary sediments were also recorded.

Complete autopsies were carried out and tissues were fixed in buffered neutral formalin (BNF) and 4% formaldehyde–1% glutaraldehyde (GF) solution (McDowell & Trump, 1977). In both the control group and the 300-ppm group two animals were fixed by intracardiac perfusion with GF. The bladders and lungs of all of the animals were inflated with BNF. The adrenal glands, thyroid glands, heart, liver, kidney, spleen, testes and brain were blotted and weighed before immersion in the fixative. The following tissues were embedded in paraffin, sectioned at 6 μm, stained with haematoxylin and eosin, and examined microscopically: adrenal glands, aorta, auditory sebaceous gland, sternal bone marrow (decalcified with RDO obtained from DuPage Kinetic Labs, Inc., Downers Grove, IL), brain and thoracic spinal cord, colon, duodenum, epididymis, oesophagus, eyes, heart, ileum, jejunum, kidney, liver, lung, lymph node (mesenteric, renal, anterior mediastinal and retropharyngeal), mammary gland, nerve (sciatic), pancreas, parathyroid, pituitary, prostate, salivary gland, seminal vesicles, skeletal muscle (semimembranosus), skin, spleen, stomach, testes, thyroid, thymus, tongue, trachea, and urinary bladder. Perl's, Van Gieson and Wilder stains (Luna, 1968) were also used on some of the paraffin-embedded tissue sections. GF-fixed liver and kidney tissue from animals treated with 0 or 300 ppm CHEB was embedded in Epon 812 (Shell Chemical Co., New York), cut at 1 μm with glass knives, and stained with toluidine blue. Impression smears were prepared from femoral bone marrow and enlarged lymph nodes and were stained with Giemsa stain.

Measurements on blood and urine, body weights and organ weights were analysed by one-way analysis of variance. Means were compared by Duncan's multiple range test (Duncan, 1955). Log transformed data were used whenever the within-group variation was proportional to the arithmetic mean and if the transformation resulted in greater homogeneity of variance among groups. Where appropriate, other data were analysed by Fisher's Exact Test for 2 × 2 tables (Bliss, 1967).

RESULTS

Mean body weights were inversely related to the concentrations of CHEB in the diet throughout the course of the study. In rats fed 150 or 300 ppm CHEB, mean body weights were always significantly

less than those of the controls. In the 75-ppm group, mean body weights were significantly less than those of the controls only between days 44 and 64. Feed consumption generally reflected growth, with significantly decreased values occurring in the 300-ppm group throughout the study.

Despite the decreased feed intake, the overall mean daily dose of CHEB ingested per kg body weight was proportional to the concentration of CHEB in the diet and was 5·4, 10·6 and 21·9 mg/kg body weight/day for the 75-, 150- and 300-ppm test groups, respectively, while the mean total amount of CHEB ingested per rat was 124, 227 and 269 mg, respectively. One rat out of the six given 300 ppm CHEB in the diet gradually lost weight after 44 days of treatment, became moribund and was killed and autopsied on day 73.

Studies of blood samples taken at the end of the test showed a statistically significant decrease in circulating erythrocytes in the 150- and 300-ppm groups, along with small, significant increases in erythrocyte haemoglobin content. Packed cell volume was inversely related to CHEB concentration and was significantly decreased in the 300-ppm group. Platelet counts appeared to increase with increasing CHEB in the diet; however, differences among group means were not statistically significant. A slight elevation (not significant) in the leucocyte count for the 300-ppm group was accompanied by a statistically significant shift ($P < 0·01$) in the relative numbers of lymphocytes to neutrophils. Compared to control values, lymphocytes were decreased while neutrophils were increased. In addition, the absolute number of eosinophils was significantly elevated ($P < 0·01$) in this group. There were numerous significant differences in plasma- and serum-constituent concentrations between the control rats and those fed 300 ppm CHEB. In the latter group, glucose, albumin, albumin:globulin ratio and urea nitrogen were all decreased, whereas alkaline phosphatase, glutamic–oxaloacetic transaminase, glutamic–pyruvic transaminase, ornithine carbamyl transferase and total bilirubin were increased. There were no significant differences in the concentrations of plasma and serum constituents between the rats fed the two lower CHEB levels and the controls.

Urine taken during the last week of the study from rats fed 300 ppm CHEB contained small amounts of blood. The incidence of blood in the urine of this group was significantly increased ($P < 0·05$, Fisher's exact test) compared with the controls. Similarly, glucose was also present in significant ($P < 0·005$) amounts in the urine of the 300-ppm group (at levels estimated to be 1–1·5 g/100 ml). Other urinary factors were not noteworthy.

In rats given 300 ppm CHEB, there were statistically significant decreases in all absolute organ weights. In the other two groups of rats fed CHEB, only heart weight in the 150-ppm group and adrenals weight in the 75-ppm group were significantly lower than control values. These differences in absolute organ weights were considered to be largely related to changes in body weight rather than to any specific effects of CHEB treatment. Similarly, relative organ weights (expressed as a simple ratio to body weight) did not indicate any specific effects of CHEB; they generally increased with increasing concentration of CHEB in the diet and were all (including relative brain weight) significantly elevated in the 300-ppm group. An allometric adjustment of organ weights to a common body weight using growth constants (Trieb, Pappritz & Lützen, 1976) helped to remove the influence of body weight. With this approach, the weights of kidneys, heart, thyroid, and brain were essentially uniform throughout all of the treated groups and were not significantly different from control values. However, allometrically adjusted weights of the liver and the spleen, and particularly of the testes, showed statistically significant increases apparently in response to dietary CHEB. Adrenals weight in the low-dose group (75 ppm) was less than that of the control and high-dose (300-ppm) groups.

Apart from the alterations in body condition associated with poor growth, gross lesions were limited to the livers of the CHEB-fed animals and the testes of animals in two of the groups. The affected livers were firmer and had irregular capsular surfaces. One animal in the 300-ppm group had bilateral diminution of testicular size with softening, while one control animal had unilateral testicular discoloration and softening. Histological lesions associated with CHEB ingestion occurred in the liver, kidney and pancreas (Table 1). Livers of rats in the 300-ppm group were severely affected and were characterized by varying combinations of bile-duct hyperplasia, fibrosis, megalocytosis, disruption of lobular architecture and individual hepatocyte necrosis. The bile-duct hyperplasia usually involved portal areas, although the hyperplasia in well developed lesions involved regions as large as several hepatic lobules. The ducts, usually lined by simple cuboidal epithelium, were often surrounded by dense fibrous connective tissue lightly

Table 1. *Lesions in rats associated with the ingestion of CHEB for 90 days**

Dose of CHEB (ppm)	No. of rats with lesions of the Liver: Bile-duct hyperplasia and fibrosis	Liver: Megalocytosis	Kidney (nephrocytomegaly)	Pancreas (acinar cell karyomegaly)
0	0	0	0	0
75	2	0	3	0
150	5	1	6	0
300	6	5	6	3

CHEB = *erythro-* and *threo-2S*-1-cyano-2-hydroxy-3,4-epithiobutane

*There were six rats in each treatment group. One rat in the 300-ppm group became moribund and was killed on day 73.

infiltrated with lymphocytes and macrophages, the latter occasionally bearing haemosiderin. In the large areas of bile-duct proliferation, the lumina tended to be larger and the epithelial lining flattened, presenting a pseudovascular pattern at low magnifications (Fig. 1a). These larger areas of biliary hyperplasia were frequently present in subcapsular locations. Tracts of proliferating biliary tissue, sometimes appearing as less differentiated structures without lumina, were composed of cells with oval, vesicular nuclei and often extended from portal to central areas resulting in the formation of pseudolobules and further architectural disruption (Fig. 1b).

Megalocytic hepatocytes occurred primarily in periportal areas, although in severely affected livers the distribution was more diffuse. The megalocytes, some of which were up to 30–40 μm in diameter, had homogeneous, basophilic cytoplasm, and enlarged, vesicular nuclei up to 20 μm in diameter with multiple, large nucleoli (Fig. 1c). Border zones between adjacent megalocytes were frequently quite distinct due to a reduced stain affinity of peripheral cytoplasm. Individually necrotic hepatocytes, which were never numerous, tended to occur in portal areas and occasionally mitotic figures were also seen.

Bile-duct hyperplasia, limited to portal areas, was the major alteration in the less severely affected livers. In a few areas of the less affected livers, megalocytosis seemed to predominate over the bile-duct lesion.

The renal lesion was characterized by well developed cytomegaly, consisting of both nuclear and cytoplasmic enlargement of proximal tubular epithelial cells throughout the cortex and outer medullary stripe (Fig. 2a). The most striking aspect of the alteration was karyomegaly, with some nuclear diameters as large as 20 μm. In the control group, proximal tubular epithelial nuclei were approximately 7–9 μm in diameter (Fig. 2b), whereas in affected animals nuclear diameters in excess of 9 μm were common, with most falling in the range of 9–11 μm. Some of the large nuclei had eosinophilic 'inclusion bodies' distinctly outlined against the nucleoplasm that were characteristic of cytoplasmic invaginations. Other morphological characteristics of the kidney appeared normal.

Pancreatic alterations were seen in half of the high-dose rats and consisted of karyomegaly of acinar cells (Table 1). While unaffected acinar cell nuclei were approximately 6–9 μm in diameter, the diameters of affected nuclei were consistently 9–11 μm and occasionally 14–15 μm.

Histologically, the abnormal testes detected grossly were characterized by degeneration of seminiferous epithelium and tubular atrophy. Because one animal bearing this testicular lesion was in the control group and the other was in the high-dose group, these lesions were considered incidental findings. The testes of all of the other animals were histologically normal.

DISCUSSION

Feeding young male rats CHEB for 90 days results in a number of dose-dependent pathological alterations. Liver and kidney lesions were present even at the 75-ppm dose level, and increased in severity and incidence at the 150- and 300-ppm levels. The kidney appeared to be more sensitive to the effects of CHEB since the fully developed lesion was present in both the 150- and 300-ppm groups. Associated with the presence of well developed liver lesions were elevations of serum alkaline phosphatase, glutamic–oxaloacetic transaminase, glutamic–pyruvic transaminase, ornithine carbamyl transferase and total bilirubin, indicating both hepatocellular damage and cholestasis. The reduced levels of blood glucose, albumin, and urea nitrogen, the altered albumin: globulin ratio, and the decreased erythrocyte and packed-cell-volume values in the high dose group were probably related to the low nutritional status of the animals. The reason for the increased corpuscular haemoglobin content is not evident. The haematuria and glucosuria observed in the high-dose group were probably not related to the nephrocytomegaly, since animals in the 75- and 150-ppm groups had nephrocytomegaly but no urinary alterations.

The renal and hepatic lesions present in these rats are remarkably similar to those seen in both aflatoxin and pyrrolizidine alkaloid toxicosis. Subacute aflatoxicosis in rats is characterized by biliary and oval cell proliferation, distortion of lobular architecture, and hepatocytic karyomegaly (Newberne & Butler, 1969). Renal tubular karyomegaly is produced by aflatoxin B_1 (Butler, 1964) and to a greater extent by aflatoxin G_1 (Butler & Lijinsky, 1970). Both agents produce liver tumours and aflatoxin G_1 is a more potent renal carcinogen than aflatoxin B_1. Hepatocellular basophilic foci seen early in livers of aflatoxin-treated rats (Newberne, 1976), and thought by some to be significant with respect to liver tumour development (Newberne, 1976; Squire & Levitt, 1975), were not observed in rats fed CHEB. Chronic ingestion of pyrrolizidine alkaloids results in hepatic megalocytosis, bile-duct proliferation and fibrosis (Bull, Culvenors & Dick, 1968), and renal karyomegaly in intoxicated swine and rats. The pattern of the karyomegaly in rats varies with the specific alkaloid; some primarily affect proximal tubules while others affect distal tubules and glomeruli (McLean, 1970).

Other agents that cause renal tubular karyomegalic alterations include alkali-treated soya protein and lysinoalanine (Woodard & Short, 1977), *S*-dichlorovinyl-L-cysteine (Terracini & Parker, 1965), 4'-fluoro-4-amino diphenyl (Mathews & Walpole, 1958), dimethylnitrosamine (Zak, Holzner, Singer & Popper, 1960), lead (Mathews & Walpole, 1958), certain gold compounds (Payne & Saunders, 1978), and the flame-retardant, tris-(2,3-dibromopropyl) phosphate (Reznik, Ward, Hardisty & Russfield, 1979). The last five substances are renal carcinogens.

Karyomegaly of pancreatic acinar cells occurred in only half of the rats in the high-dose group and appeared to be an effect of CHEB. The nature of the lesion was unclear, but it was similar to the liver and kidney changes in that there appeared to be an alteration in nuclear regulatory processes. That the pancreatic acinar cell karyomegaly is related to the reduced nutritional status of the high-dose animals, and not directly to CHEB, is possible, but unlikely, since the usual alteration associated with nutritional deprivation is acinar cell atrophy (Jubb & Kennedy, 1970).

The glucosinolate hydrolysis product, goitrin, was not present in this diet, and thyroid lesions were not

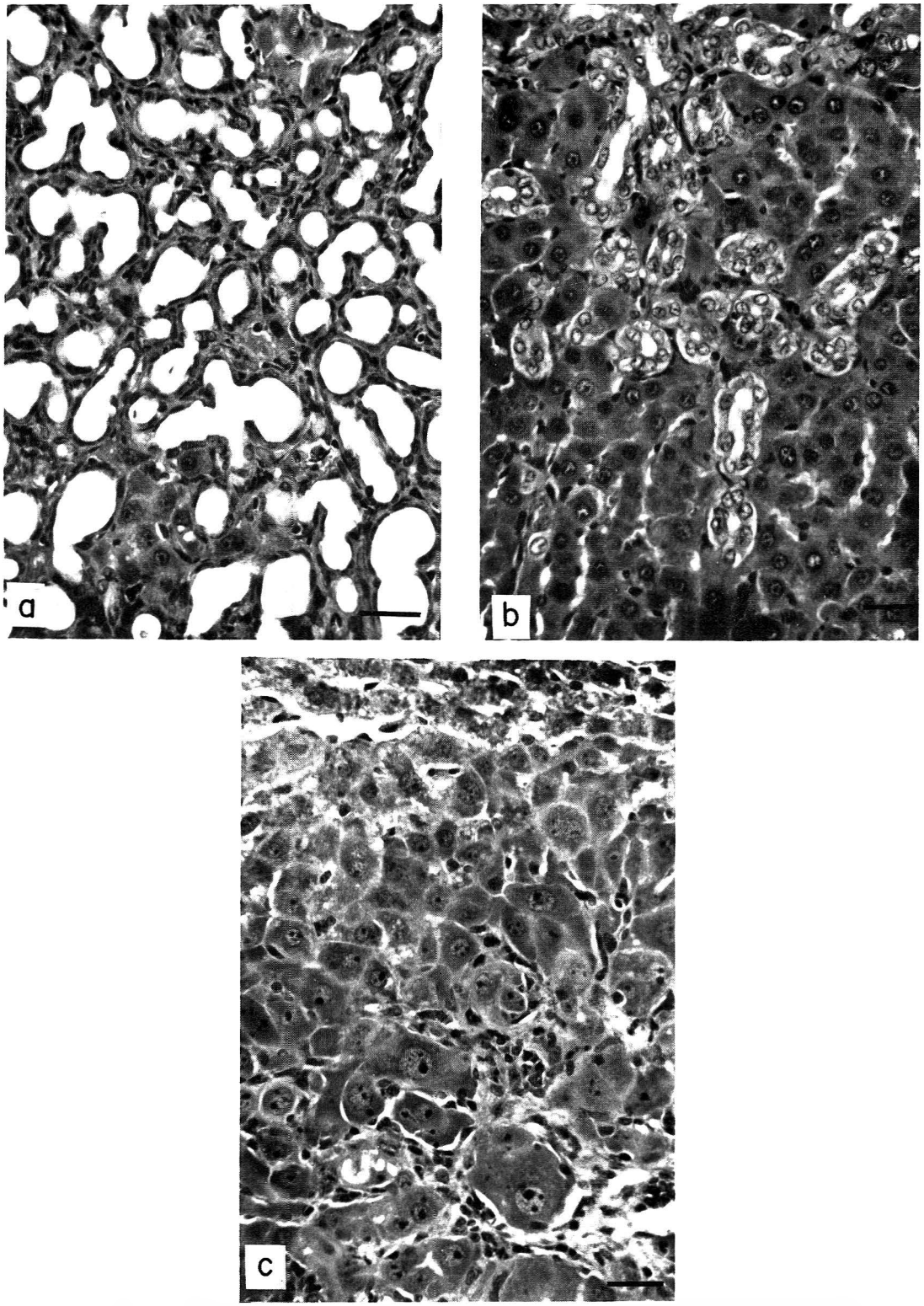

Fig. 1. Liver sections from rats given 300 ppm *erythro-* and *threo*-2*S*-1-cyano-2-hydroxy-3,4-epithiobutanes showing: (a) a large area of bile-duct proliferation with flattened epithelium and dilated lumina; (b) the proliferation of small bile ducts from the portal area (top) into the lobule parenchyma; (c) megalocytic hepatocytes, fibroplasia and light mononuclear cell infiltration adjacent to the portal area—the centrilobular area (top) shows normal hepatocytes. Each section was stained with haematoxylin and eosin and the length of each bar represents 30 μm.

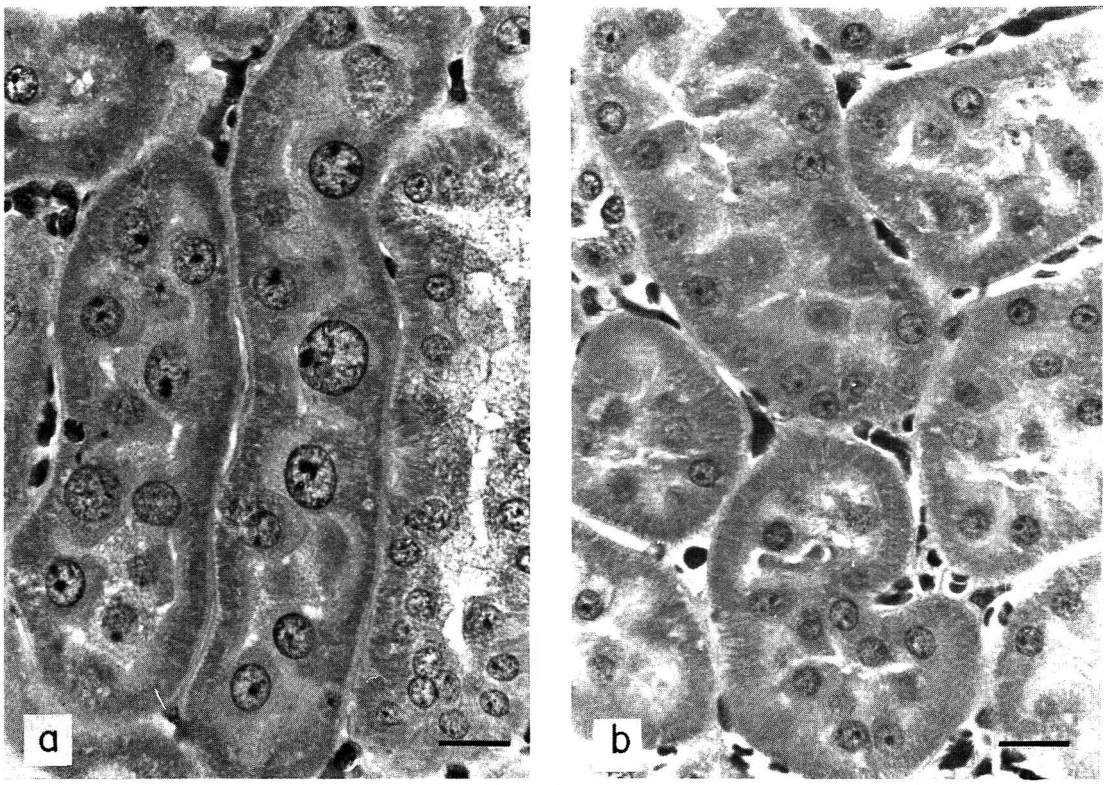

Fig. 2. Sections of the kidney of (a) a rat given 300 ppm *erythro-* and *threo-2S*-1-cyano-2-hydroxy-3,4-epithiobutanes and (b) a control rat. Note the marked enlargement of the nuclei and cytoplasm in the kidneys of the treated rat compared with the size and uniformity of those of the control. Each section was stained with haematoxylin and eosin and the length of each bar represents 20 μm.

detected. It is not clear what relationship there is between the hepatic lesions described in this study and those that are found in poultry fed rapeseed products and which are presumed to be caused by hydrolysis products of glucosinolates. The lesions induced in the poultry were characterized by centrilobular fibrosis and hepatocellular degenerative changes (Umemura, Yamashiro, Bhatnagar, Moody & Slinger, 1977).

It should be noted that the lesions present in rats fed CHEB were similar in many respects to those that preceded tumour development in animals after exposure to a number of carcinogenic agents. However, the significance of the CHEB-induced lesions is unclear and knowledge of the consequences of these potentially widespread, naturally occurring substances awaits additional study.

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COMPARATIVE INDUCTION OF ARYL HYDROCARBON HYDROXYLASE ACTIVITY *IN VITRO* BY ANALOGUES OF DIBENZO-*p*-DIOXIN

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Abstract—Dibenzo-*p*-dioxin, 23 halogenated dibenzo-*p*-dioxin analogues of known chemical purity and a predioxin (4,5,6-trichloro-2-(2,4-dichlorophenoxy)phenol) were tested for their ability to induce aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH) activity in rat hepatoma cell cultures. Plates containing 24-hr cultures were challenged for 72 hr with picomole quantities of the test compound dissolved in dimethylsulphoxide. The plates, with approximately 4×10^6 cells, were scraped and AHH activity was determined in homogenized cell extracts by conversion of benzo[*a*]pyrene to its hydroxylated derivatives. Specific enzyme activity was expressed as pmol product formed/mg protein/min at 37°C. Limits of detection, quantitation and experimental variability were determined and dose-response curves of AHH activity were plotted. The dose that produced 50% of the maximum enzyme activity (the ED_{50}) was determined for the reactive dibenzo-*p*-dioxin compounds. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) being used as the standard compound in each experiment. The average ED_{50} for TCDD was 1·54 pmol/plate and the limit of detection was 0·08 pmol/plate. The reactive dioxins, in order of decreasing biological potency, were TCDD (2,3,7,8-Cl_4-) > 2,3,7,8-Br_4- > 1,2,3,4,7,8-Cl_6- > 1,2,3,7,8-Cl_5- > 1,2,3,4,7-Cl_5- > 1,3,7,8-Cl_4- > 1,2,3,6,7,8-Cl_6- > 1,2,3,7,8,9-Cl_6- > 1,2,3,4,6,7,8-Cl_7-dibenzo-*p*-dioxin. The next order of responsiveness included 2,3,7-Cl_3- > 1,2,3,8-Cl_4- > Cl_8- (99·2%) > 1,2,3,4,6,7,9-Cl_7- > Cl_8- (99·88%) > 1,2,3,6,7,9-Cl_6- > 1,2,4,7,8-Cl_5-dibenzo-*p*-dioxin. Compounds inactive at the highest concentration tested included the predioxin and dibenzo-*p*-dioxin, and 1,3-Cl_2-, 1,6-Cl_2-, 2,3-Cl_2-, 2,7,-Cl_2-, 2,8,-Cl_2-, 1,2,3,4-Cl_4-, 1,3,6,8-Cl_4- and 1,2,4,6,7,9-Cl_6-dibenzo-*p*-dioxin. Induction of AHH activity in rat hepatoma cell cultures may provide a sensitive and reliable screening method for detecting minute amounts of certain planar polyhalogenated organic compounds suspected of contaminating food extracts and environmental samples. Chemical analysis could confirm the identity of the contaminants.

INTRODUCTION

The presence of the extraordinarily toxic substance, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and its toxic analogues in the food supply, together with polychlorinated dibenzofurans and biphenyls, represents a potential health hazard to man. TCDD is produced as an unwanted contaminant during the synthesis of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Because of the widespread use of 2,4,5-T in herbicidal applications, direct or indirect contamination of foods and animal feeds is a distinct possibility.

Baughman & Meselson (1973) reported that TCDD was found in samples of fish and crustaceans taken from locations in South Vietnam near areas heavily exposed to 2,4,5-T. Studies of the chick oedema disease have identified several dioxin isomers, including TCDD, in tissues of chickens after addition to feed of vegetable oil-by-product fatty acids derived from tallows contaminated with chlorophenol preparations (Firestone, 1973). More recently (Firestone, 1977), residues of polychlorinated dibenzo-*p*-dioxins and dibenzofurans have been found in commercial gelatin samples and have been attributed to the use of raw materials contaminated with commercial pentachlorophenols (PCP). Goldstein, Friesen, Linder, Hickman, Hass & Bergman (1977) have shown that a number of adverse changes in rat liver could be attributed to the presence of biologically active chlorinated dioxins and dibenzofurans in technical-grade PCP. Another example of inadvertent contamination of the food supply occurred in Japan when rice oil containing high levels of commercial polychlorinated biphenyls (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1971) and polychlorinated quaterphenyls (Miyata, Kashimoto & Kunita, 1978), as well as polychlorinated dibenzofurans (Morita, Nakagawa, Akiyama, Mimura & Isano, 1977), poisoned about 1000 people and produced severe clinical symptoms called 'Yusho' disease (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972).

The toxic effects of TCDD and related compounds have been well documented (Huff & Wassom, 1974; Kimbrough, 1974; McConnell, Moore, Haseman & Harris, 1978). Several adverse features of TCDD include an oral LD_{50} in the μg/kg range for many animal species, its chemical stability and lipophilic nature, and a wide variety of clinical manifestations such as porphyria, chloracne, anorexia and hepatotoxicity (Delvaux, Verstraete, Hautfenne, DeSart &

Goffin, 1975; Kimbrough, 1974). In rats, TCDD accumulates primarily in the liver and fat (Rose, Ramsey, Wentzler, Hummel & Gehring, 1976) and induces drug-metabolizing enzymes in the liver, kidney and lung (Aitio & Parkki, 1978). TCDD also elevates the activities of hepatic microsomal monooxygenases such as aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH) in both maternal and foetal livers of rats (Berry, Zachariah, Namkung & Juchau, 1976; Hook, Haseman & Lucier, 1975; Lucier, Sonawane, McDaniel & Hook, 1975).

Wassom, Huff & Loprieno (1977) reviewed the genetic toxicology of chlorinated dibenzo-*p*-dioxins, but reports about the potential mutagenicity of these compounds in several systems were inconclusive. However, evidence has accumulated that indicts TCDD as an inducer of some types of neoplasm in rats. Kociba, Keyes, Beyer, Carreon, Wade, Dittenber, Kalnins, Frauson, Park, Barnard, Hummel & Humiston (1978) reported an increased incidence of hepatocellular carcinomas and squamous-cell carcinomas in some tissues and a reduced incidence of tumours in other tissues of rats fed TCDD at 0·1 μg/kg body weight/day for 2 yr, but observed no effects in rats fed 0·001 μg/kg/day. An epidemiological study in Sweden correlated the incidence of soft-tissue sarcoma in man with exposure to phenoxyacetic acids and chlorophenols containing TCDD (Hardell & Sandström, 1979). Kouri, Rude, Joglekar, Dansette, Jerina, Atlas, Owens & Nebert (1978) reported that TCDD showed co-carcinogenic activity in inducing tumours initiated with 3-methylcholanthrene (MCA) in genetically 'non-responsive' mice and suggested that the coincident rise in AHH activity might have been a contributing factor.

In studying the biochemical effects of TCDD, many investigators have noted the potent inductive effects on AHH and the concomitant formation of cytochrome P_1-450 (*P*-448) in mammalian and avian tissues (Atlas, Thorgeirsson, Boobis, Kumaki & Nebert, 1975; Haugen, Coon & Nebert, 1976; Kitchin & Woods, 1978; Owens & Nebert, 1975 & 1976; Poland & Glover, 1973, 1974 & 1977; Poland, Glover, Robinson & Nebert, 1974; Robinson, Felton, Levitt, Thorgeirsson & Nebert, 1975). Additionally, considerable research has been directed toward the study of the genetic regulation of AHH induction by polycyclic aromatic hydrocarbons, drugs and other planar polychlorinated organic molecules such as TCDD. These studies, both *in vivo* and *in vitro*, have attempted to elucidate the complexity of mammalian membrane-bound multicomponent enzyme systems (Kouri & Nebert, 1977; Kouri, Ratrie, Atlas, Niwa & Nebert, 1974; Nebert & Felton, 1976; Nebert, Levitt, Orlando & Felton, 1977; Nebert, Robinson, Niwa, Kumaki & Poland, 1975; Niwa, Kumaki, Nebert & Poland, 1975b; Poland & Glover, 1975; Poland *et al.* 1974; Thomas, Kouri & Hutton, 1972; Thorgeirsson & Nebert, 1977).

Benedict, Gielen, Owens, Niwa & Nebert (1973) surveyed a number of mammalian cell cultures for AHH inducibility by MCA. They found a rat hepatoma cell line derived from the Reuber hepatoma with the properties of low basal AHH activity and high polycyclic aromatic hydrocarbon inducibility. Niwa, Kumaki & Nebert (1975a) subsequently demonstrated the extreme sensitivity of this rat hepatoma cell line to TCDD and proposed its use for detecting minute amounts of various foreign compounds that are inducers of AHH activity.

The purpose of our investigation was to develop a rapid, reliable and sensitive biological detection system *in vitro*. This system was intended to screen large numbers of specially prepared food extracts for suspected toxic chlorinated dioxins and related compounds prior to chemical confirmation. Suspect foods would be likely to contain mixtures of dibenzo-*p*-dioxins, dibenzofurans and polychlorinated phenolic compounds. Therefore, analysis of the relative biological potency of toxic and non-toxic dioxin analogues and other related compounds in comparison with TCDD was essential for the successful implementation of such a bioassay system. In this study, 23 dioxin analogues of known chemical purity were screened, as well as dibenzo-*p*-dioxin itself and a predioxin (4,5,6-trichloro-2-(2,4-dichlorophenoxy)phenol). The bioassay measured the induction of AHH activity in rat Reuber hepatoma cell cultures. The dose that produced 50% of the maximum enzyme activity was determined for each compound and the biological potency was compared with the standard TCDD response.

EXPERIMENTAL

Materials. The structure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is shown in Fig 1. Table 1 summarizes the purity of the various dibenzo-*p*-dioxin analogues and lists references providing information on their synthesis. Knowledge of the chemical purity of these compounds was important because minor components could be responsible for the inducing activity (Poland & Glover, 1973). Professor C. Rappe, University of Umea, Umea, Sweden, provided a sample of 4,5,6-trichloro-2-(2,4-dichlorophenoxy)phenol, a predioxin.

The tissue-culture materials, which included Eagle's Minimal Essential Medium with Earle's Salts (MEM), calf serum, foetal calf serum, penicillin and streptomycin, were supplied by Flow Laboratories, Rockville, MD. The H-4-II-E cell line was derived from Reuber H-35 hepatoma (Reuber, 1961) and has been maintained in continuous culture since 1964 (Pitot, Peraino, Morse & Potter, 1964), latterly also by Dr. E. Brad Thompson (National Cancer Institute, Bethesda, MD). The properties of the H-4-II-E tumour-cell system were described by Evans & Kovacs (1977). The rat hepatoma cell line was a gift from Dr. D. W. Nebert (National Institute of Child Health and Human Development, Bethesda, MD). The 3-hydroxybenzo[*a*]pyrene was kindly provided by Dr. H. V. Gelboin (National Cancer Institute, Bethesda, MD) and the quinine sulphate standard by R. Sonoff (American Instrument Co., Inc., Silver Spring, MD). All solvents used were of spectral-purity grade;

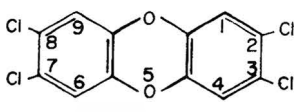

Fig. 1. Structure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Table 1. *Identity and purity of dibenzo-p-dioxin test compounds and a dioxin precursor*

Compound		Purity (%)	Ref.*
Dibenzo-*p*-dioxin		Recrystallized	D
DCDD,	1,3-	>99·0†‡	B
	1,6-	>98·0†	E
	2,3-	>99·0†	D
	2,7-	98·0†	D
	2,8-	>99·0†	D
TrCDD	2,3,7-	73·0 (+27·0% 2,7-DCDD)†	B
TCDD,	1,2,3,4-	97·0†	D
	1,2,3,8-	80·0 (+20·0% other TCDD and a PCDD)§	E
	1,3,6,8-	>98·0†‡	D
	1,3,7,8-	>99·0†‡	B
	2,3,7,8-	99·5†‡	B
PCDD,	1,2,3,4,7-	93·3 (+4·6% 1,2,3,4,7,8-HCDD and 0·2% 1,2,3,4,6,7,8-HpCDD)†	D
	1,2,3,7,8-	97·6‡	A
	1,2,4,7,8-	98·8†‡	A
HCDD,	1,2,3,4,7,8-	98·5 (+3·5% 1,2,3,4,7-PCDD)†	D
	1,2,3,6,7,8-	98·3†‡	A
	1,2,3,6,7,9-	88·3 (+8·0% PCDDs and 1·5% TCNB)†‡	A
	1,2,3,7,8,9-	98·8 (+0·1% 1,2,3,6,7,8-HCDD)†‡	A
	1,2,4,6,7,9-	91·0 (+2·0% 1,2,3,4,6,7,9-HpCDD)†‡	D
HpCDD,	1,2,3,4,6,7,8-	97·2 (+0·2% 1,2,3,6,7,8-HCDD, 0·1% 1,2,3,7,8,9-HCDD, 0·2% other HCDDs and 0·5% hexachloro-nitro- isomer)†‡	A
	1,2,3,4,6,7,9-	90·6 (+7·4% HCDDs and 0·4% TCNB)†‡	A
OCDD,	1,2,3,4,6,7,8,9-	99·2 (+0·05% 1,2,4,6,7,9-HCDD, 0·5% 1,2,3,4,6,7,9-HpCDD and 0·2% 1,2,3,4,6,7,8-HpCDD)†	D
		99·88 (+0·08% 1,2,3,4,6,7,9-HpCDD and 0·04% 1,2,3,4,6,7,8-HpCDD)†	D
TBDD,	2,3,7,8-	>98·0‡	B
TrCDP,	4,5,6-	>98·0‡	C

DCDD = Dichlorodibenzo-*p*-dioxin TrCDD = Trichlorodibenzo-*p*-dioxin
TCDD = Tetrachlorodibenzo-*p*-dioxin PCDD = Pentachlorodibenzo-*p*-dioxin
HCDD = Hexachlorodibenzo-*p*-dioxin TCNB = Tetrachloronitrobenzene
HpCDD = Heptachlorodibenzo-*p*-dioxin OCDD = Octachlorodibenzo-*p*-dioxin
TBDD = Tetrabromodibenzo-*p*-dioxin
TrCDP = Trichloro-2-(2,4-dichlorophenoxy)phenol

*References: (A) Gray, Cepa & Cantrell, 1975; (B) Kende, Wade, DeCamp, Ridge & Poland, 1974; (C) Nilsson, Andersson, Rappe & Westermark, 1974; (D) Pohland & Yang, 1972; (E) Vinopol, Yamamoto & Cassida, 1973.

†Analysis by electron-capture gas chromatography (1·2% Silar 10C on 80–100 mesh Chromosorb W-HP; column temperature (isothermal) 160–200°C; Hewlett-Packard 5713A gas chromatograph).

‡Analysis (flame-ionization gas chromatography) by supplier.

§Analysis by combined gas chromatography–mass spectrometry (Finnigan Model 3300F), using a 61 × 22-mm ID column with 3% OV-101 on 80–100 mesh Supelcoport; temperature programmed at 4°/min between 210 and 240°C.

dimethylsulphoxide (DMSO) was obtained from Burdick and Jackson Laboratories, Muskegon, MI, and isooctane, acetone, hexane and *o*-xylene were obtained from Fisher Scientific Co., Silver Spring, MD. The benzo[*a*]pyrene, NADH and NADPH were purchased from Sigma Chemicals, St. Louis, MO, and crystalline bovine serum albumin was purchased from Calbiochem, San Diego, CA.

Cell culture. The rat hepatoma cells were maintained as a continuous cell line and grown in MEM containing 10% foetal calf serum, 10% calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified 4%-CO_2 atmosphere at 37°C. The combination of both foetal calf serum and calf serum produced conditions for highly inducible AHH activity with this cell line (Owens & Nebert, 1976). For

long-term storage, cell stocks were maintained in the vapour phase of liquid nitrogen. For each experiment, confluent monolayers were trypsinized (0·25% trypsin), collected and counted with a Coulter counter. Cells were plated at a density of 10^6 cells in 4 ml of medium in 60 × 15-mm culture dishes. After incubation for 24 hr, the cultures were semiconfluent and the freshly prepared test chemical, in an appropriate solvent system, was added to each plate (0·1 ml/plate). After further incubation for 24, 48 or 72 hr, cells were washed four times with cold Dulbecco's phosphate-buffered saline and were then collected by scraping into 8 ml chilled phosphate–saline and centrifuged at 500 **g** for 10 min at 4°C. The pellets were stored frozen at −120°C until the bioassay could be performed.

Homogenization was carried out in 0·4 ml 0·25 M-sucrose–0·05 M-Tris buffer, pH 8·0, with the use of individual tightly fitting Potter–Elvejhem glass–glass homogenizers (1 ml capacity). Samples were taken for enzyme analysis (100 μl) and for protein determination (20 μl). The homogenate volume was sufficient for duplicate samples and 3–6 plates were analysed per chemical dilution. Since the statistical variability between plates was greater than the variability between enzyme determinations within plates, single enzyme analyses per plate were performed with replicate plates.

Preparation of chemicals. Except where noted, all dioxin analogues were initially dissolved in isooctane. Dilutions were prepared with DMSO and water and were applied immediately to the cell cultures; the final concentration of DMSO in the culture medium was 0·25%. The following chemical–solvent systems had no discernible effects on cell number, AHH activity or cellular protein when applied to cell cultures in 0·1-ml aliquots: DMSO–water, 1:9 v/v; DMSO–water, 1:1 v/v; acetone–water, 1:1 v/v; acetone–water–DMSO, 1:2:1 by vol.; water–DMSO–*o*-xylene, 1:2:1 by vol.; isooctane alone. Extreme caution was taken to prevent photodecomposition of the chemicals.

Enzyme assays. Aryl hydrocarbon hydroxylase activity was measured by the method of Nebert & Gelboin (1968). Test tubes, 16 × 75 mm, replaced the usual 50-ml Erlenmeyer flasks in the bioassay to facilitate the execution of approximately 200 assays/day. All glassware used in the bioassay was rinsed in deionized water and dried before use. Incubation mixtures, in a total volume of 1·0 ml, contained 50 μmol Tris–HCl (pH 8·0), 3 μmol $MgCl_2$, 0·49 μmol NADH, 0·59 μmol NADPH, 700 μg bovine albumin, 100 μl cell homogenate, and 100 nmol benzo[*a*]pyrene (substrate) added in 50 μl methanol. Incubations were carried out for 30 min at 37°C in a shaking water bath under the illumination of a General Electric F40R red bulb. The reaction was stopped by addition of 1·0 ml acetone and the mixture was shaken with 3·25 ml hexane for 10 min at 37°C. A 1·0-ml aliquot of the acetone–hexane layer was extracted with 3·0 ml N-NaOH, and the fluorescence of the aqueous fraction was measured immediately at 396 nm excitation and 522 nm emission in an Aminco–Bowman spectrophotofluorometer (American Instrument Co., Inc.). Quinine sulphate was used to check the sensitivity and accuracy of the spectrophotofluorometer. One unit of AHH activity was defined as the amount of enzyme/min at 37°C catalysing the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol 3-hydroxybenzo[*a*]pyrene standard. The specific activity was expressed in units/mg cellular protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as the standard. Enzyme activities were compared with several blanks to which acetone had been added before incubation.

Statistical analysis. An estimate of the minimum specific activity that was detectable above the control was derived in the bioassay for TCDD detection. Various methods have been suggested for calculating the lower detection limit of an assay procedure (Currie, 1968; Kaiser, 1973; Mandel & Stichler, 1957). The simplest method estimates the variability associated with background or control levels. The lower detection limit was determined from the mean value of the controls and the estimate of the control variability by using a 99% confidence interval. Enzyme activity was considered to be above the lower detection limit if a level was detected above these confidence limits. Confidence limits of three standard deviations above the control values have been suggested for 'detection limits', that is limits that can be detected as different from background but not quantified. The lower quantitation limit was determined in the same way by using a confidence limit of six standard deviations. Since the responses produced by all compounds examined were compared with the standard TCDD response in each experiment, it was important to have a reliable measure of quantitation. The ED_{50}, defined as the median effective dose producing 50% of the maximal enzyme activity, was computed by an inverse linear regression analysis for each experiment (Goldstein, 1964).

RESULTS

Induction of hydroxylase activity in response to TCDD

Figure 2 shows the results of a representative experiment in which 24-hr cultures were challenged with increasing levels of TCDD for 24, 48 and 72 hr to determine the optimal time for exposure. Results are shown in terms of specific activity and fractional response. Although maximum AHH induction was obtained at 24 hr with the higher doses of TCDD, responses to the smaller doses were not great enough to provide adequate sensitivity. Therefore, the exposure time of 72 hr was selected for subsequent experiments. The basal AHH activity in solvent-control plates ranged from 0·5 to 1·15 units/mg protein/min.

The mean fractional responses and standard deviations of specific AHH activity were plotted as a function of TCDD concentration (pmol/plate or $-\log_{10}$ molar) for 17 experiments (Fig. 3). Evaluation of the fractional response was used to compare the induction of hydroxylase activity between experiments because the absolute maximum specific activity varied from 40 to 120 units/mg protein/min for the 17 experiments. By calculating the fractional response in each experiment, the variation in maximal response between experiments could be eliminated. The concentration that produced maximal enzyme activity for each experiment was assigned a response value of 1·0. Results for all other concentrations of TCDD, as well as those of other compounds, were calculated as a fraction of this response. The mean ED_{50} for TCDD

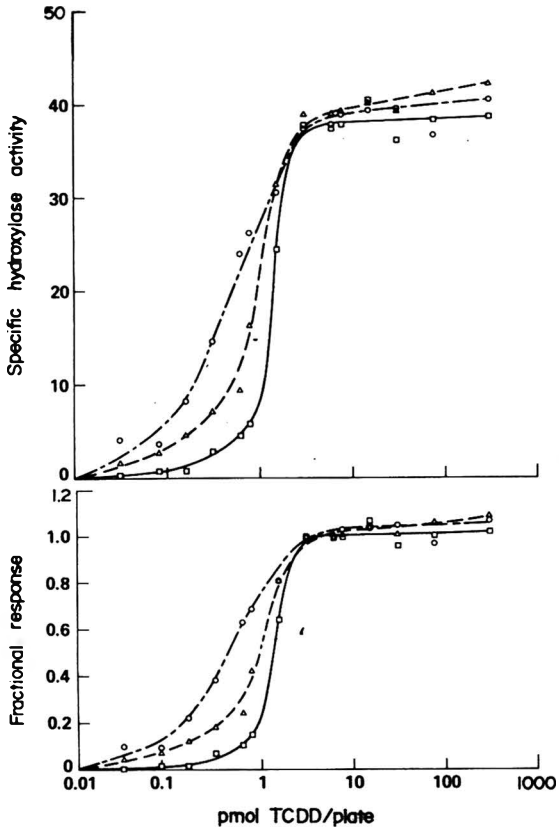

Fig. 2. Aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase specific activity and the fractional response of induction as a function of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) concentration and time at 24 hr (□), 48 hr (△) and 72 hr (○). Each point represents the average of six determinations (six separate plates).

was 1·54 pmol/plate (0·38 nM) with a range of 0·555–5·024 pmol/plate as determined by inverse linear regression analysis. We found during a year of experimentation that the TCDD standard became increasingly less potent. When a new sample of TCDD was used, the sensitivity to low doses of TCDD was again evident and a consistent dose response was produced. Extreme caution was taken to prevent contamination of the standard TCDD solution. Therefore, measured aliquots of the TCDD standard solution were stored in tightly sealed glass containers in the dark and only one such aliquot was used for each experiment. Approximately 4×10^6 cells were present in each Petri dish after a 72-hr incubation and no reduction in cell numbers was observed at the levels tested.

Results for approximately 90% of all solvent-control AHH activity determinations were less than 1·0 units/mg protein/min and the activity was often immeasurable. The induction of AHH specific activity that could be statistically detected was an increase of 3·0 units/mg protein/min above mean control activity, a threefold increase. The induction of AHH specific activity that could be statistically quantitated was an increase of 6·0 units/mg protein/min above mean control activity; this was was a sixfold increase. The detection and quantitation limits for the bioassay are also shown in Fig. 3. The statistical limit of detection was between 0·08 and 0·155 pmol TCDD/plate (25 and 50 pg, respectively), while the lower statistical limit of quantitation was above 0·31 pmol TCDD/plate (100 pg).

*Comparative induction of AHH activity by analogues of dibenzo-*p*-dioxin*

Table 2 summarizes the results of several experi-

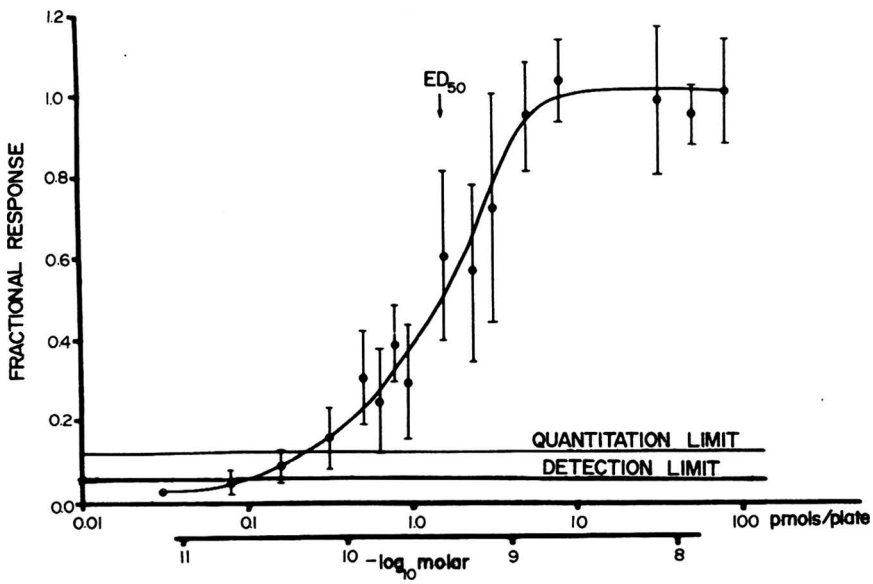

Fig. 3. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin dose-response curve. The mean fractional response and standard deviation of AHH induction is plotted as a function of TCDD concentration (pmol/plate or $-\log_{10}$ molar) for 17 experiments. The concentration that produced a maximal enzyme activity was assigned a fractional response of 1·0 and all other concentrations were normalized as fractions of this response. The ED_{50} (median effective dose producing 50% of the maximal enzyme activity) for TCDD is 1·54 pmol/plate as determined by inverse linear regression analysis (arrowed). [Reprinted from Bradlaw & Casterline, *J. Ass. off. analyt. Chem.* 1979 **62**, 904, with permission of the Association of Official Analytical Chemists.]

ments in which the ED_{50} responses of 22 halogenated congeners of dibenzo-*p*-dioxin, a predioxin and dibenzo-*p*-dioxin were compared with the ED_{50} response of TCDD. The ED_{50} values are reported as pmol/plate for each experiment. The relative biological potency for each compound was computed by assigning TCDD a biological potency of 100 and comparing the resulting ED_{50} of each compound with that of TCDD within each experiment. The ED_{50} values (pmol/plate) of TCDD from which the relative biological potencies were computed are also listed in Table 2.

Compounds that did not produce maximal AHH induction because of solubility problems or their unavailability at high concentrations were reported as projected ED_{50} values based on an inverse linear regression analysis of the slope of the curve. Compounds that did not induce hydroxylase activity at the highest concentration tested are indicated in a footnote to Table 2.

In comparison with TCDD, assigned a biological potency of 100, eight analogues of dibenzo-*p*-dioxin showed a relative biological potency greater than 0·2%. These, in order of decreasing activity, were TCDD (2,3,7,8-tetrachloro-) > 2,3,7,8-tetrabromo- > 1,2,3,4,7,8-hexachloro- > 1,2,3,7,8-pentachloro- > 1,2,3,4,7-pentachloro- > 1,3,7,8-tetrachloro- > 1,2,3,6,7,8-hexachloro- > 1,2,3,7,8,9-hexachloro- > 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin. Seven of these nine congeners have halogen substitutions in the four lateral ring positions, the exceptions being 1,3,7,8-tetrachloro- and 1,2,3,4,7-pentachlorodibenzo-*p*-dioxin. However, the response produced by the latter was probably due to the 4·6% 1,2,3,4,7,8-hexachloro- impurity in the preparation.

The next group, in decreasing order of responsiveness, included 2,3,7-trichloro- > 1,2,3,8-tetrachloro- > octachloro- (99·2%) > 1,2,3,4,6,7,9-heptachloro- > octachloro- (99·88%) > 1,2,3,6,7,9-hexachloro- > 1,2,4,7,8-pentachlorodibenzo-*p*-dioxin. Although the AHH-inducing activity may have been due to the major component in all of the above preparations (with the probable exception of 1,2,3,4,7-pentachlorodibenzo-*p*-dioxin), minor components may also have contributed to the biological responses observed. For example, the less pure octachloro- compound (99·2%) produced a biological response approximately seven times greater than the more pure (99·88%) octachloro- preparation.

Compounds that were inactive at the highest concentration tested included the predioxin, dibenzo-*p*-dioxin itself and its 1,3-, 1,6-, 2,3-, 2,7- and 2,8-dichloro-, 1,2,3,4- and 1,3,6,8-tetrachloro- and 1,2,4,6,7,9-hexachlorodibenzo-*p*-dioxin, all with two or less halogen substitutions in the lateral (2,3,7,8) ring positions.

DISCUSSION

Since cell death in the rat hepatoma cell system is not an obvious feature of this enzyme-induction test, it is difficult to ascribe the results to cytotoxicity. This finding is consistent with that of Beatty, Lembach, Holscher & Neal (1975), who reported no changes in growth rates or morphology in a variety of cell lines cultured with microgram concentrations of TCDD. However, several reports attest to the fact that halogenated analogues of dibenzo-*p*-dioxin that are potent inducers of AHH activity are also the most toxic in several animal species. Poland & Glover (1973) were among the first to note the correlation, using AHH activity in chick-embryo liver and foetal death for the comparison.

It is apparent that a structure–activity relationship exists between the location of the halogen atoms on the dibenzo-*p*-dioxin molecule and an ability to induce AHH activity in the rat hepatoma cell culture system, with TCDD being the most reactive compound tested. Dioxins with halogen atoms at the four lateral ring positions produced a greater biological response than those with halogens at three lateral ring positions, and dioxins with two halogen atoms at the lateral ring positions appeared to be inactive under the conditions of this test. The results are consistent with other investigations in which series of dibenzo-*p*-dioxins were tested. Poland & Glover (1973) found this correlation in the chick embryo and McConnell *et al.* (1978) reported similar findings when they compared the toxicity of the dibenzo-*p*-dioxins in mice and guinea-pigs. Although the degree of toxicity may vary among animal species, the relative order of biological potency of the dibenzo-*p*-dioxins, observed by measurements of toxicity or induction of AHH activity in rat hepatoma cells, appears to be the same.

Our investigation of the quantitative aspects of the enzyme induction test showed that the limit of detectability was 25 pg (0·08 pmol) TCDD/plate and the average ED_{50} response was 1·54 pmol TCDD/plate (0·38 nM). These values approximate those of Niwa *et al.* (1975a), who reported a limit of detectability of 10 pg TCDD/plate and an ED_{50} response of 0·23 nM when a slightly different protocol was used with the same rat hepatoma cell line (H-4-II-E). We have since found that when isooctane instead of DMSO was used as the solvent for TCDD applications, we achieved a limit of detectability of 10 pg TCDD/plate and an ED_{50} value of approximately 0·14 pmol/plate (Bradlaw & Casterline, 1979).

Poland, Glover & Kende (1976) have attempted to identify a hepatic cytosol species that binds TCDD and acts as the receptor for the hepatic AHH activity. Receptor binding may serve as the basis for another sensitive method to detect potent inducers of AHH; analogues of TCDD and TCDF may bind competitively with the receptor protein, resulting in diminished affinity for the inducing compound in comparison with TCDD. Guenthner & Nebert (1977) isolated a cytosol receptor from the rat hepatoma cell line H-4-II-E and found high binding affinity for TCDD compared with three other cell lines; they regarded this cell line as genetically 'responsive'. This result is similar to those findings with 'responsive' strains of mice (Poland *et al.* 1976).

The significance of the cell culture–enzyme induction test as described in this report relates to its usefulness as a screen for detecting biologically potent planar polyhalogenated organic compounds that may be present in foods and environmental samples. The correlation between AHH induction in cell cultures and known toxic responses in animal and avian species for the most potent substances adds to the importance of this simple biological detection system. Unknown samples can be tested in a matter of days, but

Table 2. *ED_{50} values (median effective dose, producing 50% of the maximal enzyme activity) for dioxins*

Compound*	ED_{50} (pmol/plate)	Relative biological potency	2,3,7,8-TCDD ED_{50} (pmol/plate)
2,3,7,8-TCDD	1·54†	100·0‡	
2,3,7,8-TBDD	3·64	71·0	2·61
	3·85	52·0	2·02
1,2,3,4,7,8-HCDD	12·43	7·0	0·91
	49·86	10·0	5·02
1,2,3,7,8-PCDD	29·46	1·9	0·56
	20·47	3·6	0·74
	15·23	22·7	3·45
1,2,3,4,7-PCDD	120·47	0·8	0·91
	107·46	4·8	5·02
1,3,7,8-TCDD	113·78	2·3	2·02
	612·16	0·3	2·61
1,2,3,6,7,8-HCDD	701·38§	0·1	0·74
	63·76	0·9	0·56
	151·25	0·9	1·34
	167·73	1·4	2·34
1,2,3,7,8,9-HCDD	84·52	0·9	0·74
	292·84	0·2	0·56
1,2,3,4,6,7,8-HpCDD	129·50	0·5	0·70
	972·13	0·3	3·45
2,3,7-TrCDD	$4·6 \times 10^3$	0·11	5·02
1,2,3,8-TCDD	$3·3 \times 10^3$	0·016	0·56
	$1·7 \times 10^3$	0·043	0·74
1,2,3,4,6,7,8,9-OCDD (99·2%)	$1·6 \times 10^3$	0·034	0·56
	$3·4 \times 10^3$	0·022	0·74
1,2,3,4,6,7,9-HpCDD	$3·8 \times 10^3$‖	0·018	0·70
	$13·9 \times 10^3$‖	0·025	3·45
	$28·2 \times 10^3$‖	0·011	3·12
1,2,3,4,6,7,8,9-OCDD (99·88%)	$>5 \times 10^3$‖	—	1·23
	$79·5 \times 10^3$‖	0·004	3·45
1,2,3,6,7,9-HCDD	$>5 \times 10^3$‖	—	5·02
	330×10^3‖	$2·2 \times 10^{-4}$	0·70
1,2,4,7,8-PCDD	130×10^6‖	$7·7 \times -10^{-7}$	0·56
Others¶	—	Inactive	—¶

*For abbreviations see Table 1.

†Mean ED_{50} value for 2,3,7,8-TCDD from 17 experiments was 1·54 pmol/plate with a range of 0·555–5·024.

‡2,3,7,8-TCDD was assigned a relative biological potency of 100%. Compounds were compared with TCDD within each experiment.

§Compound was judged to be insoluble in the isooctane solvent. Other experiments used acetone-*o*-xylene as solvent, in which 1,2,3,6,7,8-HCDD was soluble.

‖Projected ED_{50} values based on an inverse linear regression analysis of the slope of the curve.

¶Compounds that at the highest concentration tested (50×10^3 pmol/plate) showed no inducing activity were (with the TCDD ED_{50}—in pmol/plate—for the particular experiment in brackets): TrCDP (the predioxin; 0·56), dibenzo-*p*-dioxin (0·91), 1,3-DCDD (0·56), 1,6-DCDD (0·56), 2,3-DCDD (0·74), 2,7-DCDD (0·91), 2,8-DCDD (0·56), 1,2,3,4-TCDD (0·56), 1,3,6,8-TCDD (5·02) and 1,2,4,6,7,9-HCDD (5·02).

chemical analysis must provide the ultimate identification of the inducing substance in mixtures. Unknown samples can be quantitated with this system *in vitro* when TCDD, the most reactive compound, is used as a standard. The comparison allows for a judgement of the unknown inducer in terms of parts per billion or parts per trillion of TCDD. Efforts are currently under way to screen food samples by this *in vitro* procedure and to test pure chlorinated dibenzofuran and biphenyl compounds for an assessment of the relative biological potency of these substances in comparison with TCDD.

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COMPARATIVE DISTRIBUTION, EXCRETION AND METABOLISM OF DI-(2-ETHYLHEXYL) PHTHALATE IN RATS, DOGS AND MINIATURE PIGS

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Abstract—Di-(2-ethylhexyl phthalate (DEHP) was administered in the diet to male Sprague–Dawley rats, beagle dogs and miniature swine of the Hormel strain in doses of 50 mg/kg/day for 21–28 days before administration of a single dose of [^{14}C]DEHP at 50 mg/kg. The animals were then killed at various times, and tissues, organs, urine and faeces were analysed for distribution of radioactivity. Approximately 84% of the [^{14}C]DEHP radioactivity was excreted in the urine and faeces of rats during the first 24 hr; in dogs and pigs, excretion during this time was 67 and 37%, respectively. Elimination of ^{14}C was rapid in rats, slightly prolonged in dogs and least rapid in pigs; excretion in all three species was virtually complete in 4 days. Faecal excretion (75%) predominated in dogs, and urinary excretion (79%) predominated in pigs. Thin-layer chromatography showed the presence of at least four radioactive substances in rat urine, three in dog urine and five in pig urine. No more than a trace of unmetabolized DEHP was found in the urine of rats, dogs or pigs. In all three species, bile contained some metabolites that differed from those in urine, although some metabolites appeared to be common to both. Bile from rats and dogs contained a substance that, when hydrolysed under slightly acidic conditions, yielded a substance that migrated like mono-(2-ethylhexyl) phthalate in the chromatographic system used.

INTRODUCTION

Phthalate esters are used as plasticizers in the manufacture of certain food-packaging materials, and are usually ingested by humans in food that has been enclosed in these materials. Studies have been reported on the distribution, excretion and metabolism in rats of di-(2-ethylhexyl) phthalate (DEHP), the most commonly used phthalate plasticizer (Albro, Thomas & Fishbein, 1973; Daniel & Bratt, 1974; Schulz & Rubin, 1973; Williams & Blanchfield, 1974). *In vitro* work with DEHP has shown that this compound is hydrolysed by lipases (Albro & Thomas, 1973) and by esterases (Carter, Roll & Petersen, 1974), and is degraded by cells derived from the intestinal mucosa (Rowland, 1974; Rowland, Cottrell & Phillips, 1977).

When DEHP is ingested by the rat, it appears to be partially hydrolysed to the monoester, and the residual 2-ethylhexyl chain is subjected to ω and ω-1 oxidation (Albro *et al.* 1973; Daniel & Bratt, 1974). Recently, Chu, Villeneuve, Secours, Franklin, Rock & Viau (1978) investigated the metabolism and distribution of mono-2-ethylhexyl phthalate (MEHP) in Sprague–Dawley rats and found that it was extensively metabolized after oral administration. The metabolites, which were the same as those obtained from DEHP, resulted from side-chain oxidation, and appeared to indicate that MEHP was an intermediate in the metabolism of DEHP by the rat. A study of the distribution and excretion of DEHP in trout showed that much of the dose was contained in bile, and that the major metabolite in this species was MEHP glucuronide (Melancon & Lech, 1976).

Wallin, Klamer, Nicora & Thompson (1974) performed limited distribution-excretion studies in beagle dogs 24 hr after oral administration of DEHP in a dose of 60 or 300 mg/kg body weight. In our present study, we investigated the distribution and excretion of DEHP 4, 8 and 24 hr and 4 days after an oral dose of 50 mg DEHP/kg was given to male Sprague–Dawley rats. The distribution and excretion of DEHP 4 and 24 hr and 4 days after administration of an oral dose of 50 mg DEHP/kg to male beagle dogs and male Hormel miniature swine are also reported.

Urine and bile samples obtained from the experimental animals were examined for metabolites by thin-layer chromatography (TLC). These results, although they do not elucidate the molecular structure of the metabolites, are included for comparative purposes.

EXPERIMENTAL

Chemicals. DEHP was obtained from Pfaltz & Bauer, Inc., Stamford, CT; according to the supplier, the minimum ester content was 99·6%. The labelled DEHP was the di-ester of 2-ethylhexanol with phthalic acid, labelled with carbon-14 (9·74 mCi/mmol) in the carbonyl group, and was synthesized by Mallinckrodt Labeled Compounds, St. Louis, MO. Radiochemical purity was equal or to greater than 98% in the four TLC solvent systems described below.

MEHP was synthesized by a modification of the method of Pickard & Kenyon (1913). Equimolar quantities of phthalic anhydride and 2-ethylhexanol were dissolved in toluene and refluxed for 20 hr. The reaction mixture was cooled and extracted three times with M-Na_2CO_3. The aqueous layer was acidified with 3 N-H_2SO_4 and extracted three times with methylene chloride; the extraction of MEHP was complete and methylene chloride did not extract any side product

of phthalic acid. By repeated extraction of the methylene chloride solution with M-Na_2CO_3, acidification with 3 N-H_2SO_4 and re-extraction with fresh methylene chloride, a chromatographically pure product was obtained and was used as the standard for MEHP in TLC.

Animals and diets. Adult male Sprague–Dawley rats weighing 200–300 g were maintained on Purina Ground Laboratory Chow during the experiment. Pure-bred male beagles, weighing 7–10 kg and approximately 1 yr old, were maintained on Purina Laboratory Canine Diet. Male miniature swine of the Hormel strain, weighing 10–25 kg and aged between 4 months and 1 yr were maintained on SR No. 3 Diet (obtained from the US Department of Agriculture, Agriculture Research Center, Beltsville, MD); this diet contained 16% protein, 75% carbohydrate and 2·9% fat. Tap water was available *ad lib.*

Treatment. All animals were pretreated orally with 50 mg DEHP/kg body weight/day by adding the compound to the daily diet for 21–28 days; they were then fasted overnight before administration of the radioactive dose. A corn oil solution of [^{14}C]DEHP (approximately 5 μCi/kg body weight), together with an appropriate amount of unlabelled ester to bring the dosage to 50 mg/kg, was administered to rats by gastric intubation. The same dose was administered to dogs and pigs in a No. 12 or No. 13 gelatin capsule.

Metabolic experiments. Immediately after the animals were given [^{14}C]DEHP, they were placed in individual metabolism cages that permitted separate collection of urine and faeces. Administration of the DEHP-containing diets was continued until the animals were killed. Urine and faeces were assayed as previously described (Ikeda, Sapienza, Couvillion, Farber, Smith, Inskeep, Marks, Cerra & van Loon, 1978).

Sample collection and preparation. Rats were killed by cervical dislocation and exsanguination; dogs and pigs were killed by electrocution and exsanguination. Organs, tissues and bile were prepared for radioactivity assay as previously described (Ikeda *et al.* 1978).

Bile samples were obtained from the dogs and pigs at autopsy and from rats by bile-duct cannulation over a 4-hr period immediately following [^{14}C]DEHP administration. In rats anaesthetized by ip injections of 50 mg sodium pentobarbital/kg, the abdominal cavity was opened and the bile duct was cannulated with polyethylene tubing (PE-10, Clay-Adams, Parsippany, NJ). When bile flow was obtained, the rats were injected intragastrically with 50 mg [^{14}C]DEHP/kg in corn oil solution and were maintained under anaesthesia with pentobarbital supplemented with diethyl ether for a total of 4 hr. A pooled sample of bile obtained from four rats during this 4 hr period was used for the experiments.

The extraction procedure for pig, dog and rat bile was as follows. A 1-ml sample of bile was pipetted into a glass-stoppered, 15-ml centrifuge tube, and 1 ml 20% $NaHSO_4$ solution was added. The mixture was extracted three times with 5-ml portions of ethyl acetate, using a Vortex mixer. The ethyl acetate layers were sampled for radioactivity, dried with Na_2SO_4 and concentrated under nitrogen. In every case, 100% of the bile radioactivity was extracted into the ethyl acetate. The concentrated extract was examined for metabolites by TLC.

Samples of urine and faeces from dogs and pigs were homogenized in chloroform–methanol (2:1, v/v) and extracted with 0·1–0·2 mM-aqueous NaOH (pH 10). Aliquots of each of these layers were assayed for radioactivity by liquid scintillation counting.

Analyses. For TLC studies, commercially prepared 5 × 20-cm glass plates coated with 250-μm layers of silica gel GF were obtained from Analtech, Inc., Newark, DE. The solvent systems used were: (A) benzene–methanol–glacial acetic acid, 90:5:5 (by vol.); (B) chloroform–methanol–glacial acetic acid, 98:1:1 (by vol.); (C) methylene chloride (100%); (D) hexane–ethyl acetate, 9:1 (v/v). After the plates had been developed, they were examined under UV light and locations of the known compounds were marked. To determine regions of radioactivity, sequential 5-mm sections of the silica-gel layer were scraped from the origin to the front into liquid scintillation vials containing 1 ml *N*,*N*-dimethylformamide. To each vial 10 ml liquid scintillation phosphor containing (per litre) 750 ml scintillation-grade toluene, 250 ml anhydrous methanol, 5·0 g 2,5-diphenyloxazole and 0·6 g 2,2'-*p*-phenylenebis (4-methyl-5-phenyl)oxazole were added. The contents of the vial were well mixed and were counted in a liquid scintillation counter (Packard Model 3390, Packard Instrument Co., Downers Grove, IL).

Aliquots from aqueous–organic partition samples and urine samples were pipetted into low-potassium glass vials for scintillation counting. Chloroform–methanol extracts were dried after pipetting to remove the solvent and eliminate chemical quenching. After addition of 10 ml Instagel® (Packard Instrument Co.) to each vial, the samples were mixed well and counted in the liquid scintillation counter. An internal standard of [^{14}C]toluene was used to correct for quenching.

RESULTS

Distribution and excretion of [^{14}C]*DEHP*

The excretion of ^{14}C and distribution of residual ^{14}C in rats, dogs and pigs at various times after administration of a single oral dose of [^{14}C]DEHP are presented in Tables 1, 2 and 3, respectively. Approximately 84% of the administered radioactivity was excreted in the urine and faeces of rats during the first 24 hr; in dogs and pigs, excretion during this time was 67 and 37%, respectively. Excretion of ^{14}C was virtually complete in 4 days. Faecal excretion predominated in rats and dogs (53 and 75%, respectively), but urinary excretion predominated in pigs.

A substantial amount of radioactivity was present in the gastro-intestinal tract at day 1 in all species, and a small amount remained after 4 days. Bile samples from dogs, and to a lesser extent from pigs, accounted for a significant amount of the administered ^{14}C dose. In bile-duct cannulated rats, less than 1% of the administered ^{14}C dose was secreted in the bile during the 4-hr period.

Only a small amount of [^{14}C]DEHP radioactivity was distributed into body fat, the amount accounted for in this way being greatest in pigs and least in dogs. In all three species, radioactivity was negligible in the

Table 1. *Distribution and excretion of radioactivity after oral administration of di-(2-ethylhexyl) [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male Sprague–Dawley rats*

Sample	Mean values* for rats killed at			
	4 hr	8 hr	1 day	4 days
Liver	2·24 ± 0·42	1·84 ± 0·27	0·45 ± 0·09	0·02 ± 0·00
Kidney	0·19 ± 0·03	0·25 ± 0·07	+†	+†
Gastro-intestinal tract‡	82·56 ± 1·51	75·26 ± 1·92	6·03 ± 0·62	0·26 ± 0·11
Lung	+†	0·06 ± 0·01	0·03 ± 0·01	0·14 ± 0·00
Brain	0·02 ± 0·00	+†	—	—
Fat	0·44 ± 0·09	0·41 ± 0·08	0·11 ± 0·06	0·04 ± 0·00
Urine	2·78 ± 1·53	9·17 ± 1·73	27·34 ± 3·06	37·46 ± 3·46
Faeces	0·81 ± 0·33	2·45 ± 0·64	56·71 ± 3·22	53·23 ± 4·81
Recovery (% of total dose)...	88·22 ± 1·36	90·00 ± 2·96	90·60 ± 1·97	93·20 ± 3·40

*Values are mean percentages of the dose ± SEM/whole organ or total urine or faeces of six rats. Fat was estimated to be 7% of body weight.
†Present, but in amounts too small to be estimated (<0·01% of dose/g sample).
‡Including contents.

brain and minimal in the lung. Muscle tissue of dogs and pigs contained only small amounts of radioactivity. Earlier studies by Williams & Blanchfield (1974) also indicated minimal distribution of [^{14}C]DEHP radioactivity into skeletal muscle in rats.

Radioassay of blood samples obtained after administration of [^{14}C]DEHP indicated a blood elimination half-life of 1·4 hr in dogs and 3·0 hr in pigs.

Thin-layer chromatography of bile

Ethyl acetate extracts of bile samples obtained from rats by bile-duct cannulation were subjected to TLC in solvent system A. A highly polar metabolite that remained at the origin accounted for 84·8% of the ^{14}C (Table 4), and no unchanged DEHP (R_F 0·9) was observed. When the bile was incubated under mildly acidic conditions (pH 5·0, 0·1 M-acetate buffer), the peak at R_F 0 decreased by 18·5% and the substance(s) that migrated to R_F 0·7 quadrupled, indicating the presence of acid-labile conjugates of metabolites formed from DEHP.

The amount of radioactivity in bile samples obtained from dogs at autopsy was substantial at 4 hr but decreased to a smaller, yet measurable, amount at 4 days (Table 2). In addition, approximately 1·9% of the radioactive dose was found in the gastro-intestinal tract at 4 days. Quantitative TLC (solvent system A) of the pooled 4-hr and 1-day bile samples indicated only a trace (<0·1%) of unchanged DEHP. A major peak remained at the origin; a substance that migrated to R_F 0·7 and a small amount of a substance at R_F 0·5 were also found (Table 4). When the bile was incubated under mildly acidic conditions (pH 5·0, 0·1 M-acetate buffer), virtually all of the highly polar substance (R_F 0 in solvent system A) was converted to substances that migrated on the TLC plate with R_F values of 0·6 and 0·7. The substance that migrated at R_F 0·7 was indistinguishable from MEHP. These results indicate that acid-labile substances, probably conjugates, were secreted in the bile of dogs.

In bile samples obtained from pigs at autopsy, radioactivity rapidly decreased with time and was

Table 2. *Distribution and excretion of radioactivity after oral administration of di-(2-ethylhexyl) [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male beagle dogs*

Sample	Mean values* for dogs killed at		
	4 hr	1 day	4 days
No. of dogs...	4	4	5
Liver	1·58 ± 0·58	1·66 ± 0·25	0·13 ± 0·04
Kidney	0·09 ± 0·01	0·06 ± 0·00	0·01 ± 0·00
Bile	7·20 ± 1·27	9·82 ± 4·08	0·57 ± 0·16
Gastro-intestinal tract†	96·30 ± 4·20	15·85 ± 1·36	1·92 ± 0·28
Lung	0·04 ± 0·01	0·02 ± 0·00	0·01 ± 0·00
Brain	0·01 ± 0·00	0·01 ± 0·00	+‡
Fat	0·11 ± 0·02	0·11 ± 0·02	0·17 ± 0·03
Muscle	1·75 ± 0·29	0·77 ± 0·28	0·32 ± 0·12
Urine	2·34 ± 0·36	11·66 ± 1·73	20·74 ± 2·50
Faeces	−§	55·60 ± 3·66	75·07 ± 3·14
Recovery (% of total dose)...	108·99 ± 2·04	101·59 ± 2·10	99·43 ± 1·17

*Values are percentages of the administered dose ± SEM/whole organ or total urine or faeces. Fat was estimated to be 1% and muscle 46% of body weight.
†Including contents.
‡Present, but in amounts too small to be estimated (<0·01 of dose/g sample).
§No samples.

Table 3. *Distribution and excretion of radioactivity after oral administration of di-(2-ethylhexyl) [^{14}C]phthalate (50 mg/kg in corn-oil solution) to male miniature pigs*

Sample	Mean values* for pigs killed at		
	4 hr	1 day	4 days
Liver	0·94 ± 0·19	0·25 ± 0·06	0·07 ± 0·00
Kidney	0·30 ± 0·08	0·08 ± 0·02	0·02 ± 0·00
Bile	1·19 ± 0·44	0·17 ± 0·05	0·01 ± 0·00
Gastro-intestinal tract†	84·85 ± 4·37	62·33 ± 5·58	1·69 ± 0·33
Lung	0·07 ± 0·01	0·02 ± 0·00	0·01 ± 0·00
Brain	+‡	+‡	0·01 ± 0·00
Fat	0·97 ± 0·08	0·42 ± 0·11	0·70 ± 0·20
Muscle	0·65 ± 0·12	0·38 ± 0·12	0·42 ± 0·21
Urine	8·05 ± 1·68	36·97 ± 3·03	79·42 ± 7·90
Faeces	0·05 ± 0·03	0·11 ± 0·04	25·73 ± 6·93
Recovery (% of total dose)...	99·01 ± 3·79	100·78 ± 3·97	109·80 ± 2·92

*Values are percentages of the administered dose ± SEM/whole organ or total urine or faeces of five pigs. Fat was estimated to be 22·1% and muscle 36·7% of body weight.

†Including contents.

‡Present, but in amounts too small to be estimated (<0·01% of dose/g sample).

barely detectable after 4 days (Table 3). TLC examination (solvent system A) of samples obtained 4 hr and 1 day after treatment indicated a highly polar metabolite that remained at the origin (Table 4). Only a trace (<0·1%) of unchanged DEHP (R_F 0·9) was found in any of the bile samples from pigs, even at 4 hr. A small amount of a substance that was slightly more polar than MEHP (R_F 0·5 compared with R_F 0·7 for MEHP) was also found. Since biliary secretion does not appear to be a major route of excretion for DEHP in pigs, these samples were not acid-treated.

Thin-layer chromatography of urine

TLC examination of pooled (days 1 and 2) urine obtained from rats revealed four radioactive peaks when the plate was developed in solvent A (Table 4). A minor peak (7·2% of total urinary radioactivity) remained at the origin, and peaks at R_F 0·2, 0·5 and 0·6 were observed. The major peak was at R_F 0·5 (64·9% of the urinary radioactivity), and the peaks at R_F 0·2 and 0·6 were approximately equal in size (14·6 and 13·4%, respectively, of the total urinary radioactivity).

Pooled (days 1 and 2) urine obtained from dogs revealed three radioactive peaks when the TLC plate was developed in solvent A (Table 4). One peak remained at the origin, and the second and third peaks had R_F values of 0·5 and 0·6, respectively. In some of the urine samples, the peak at the origin was replaced by a peak migrating to R_F 0·7, and this spot was indistinguishable from authentic MEHP.

In pooled (days 1 and 2) urine from pigs, five radioactive peaks were typically present when the TLC plates were developed in solvent A. A minor peak (14·8% of total urinary radioactivity) remained at the origin, and peaks with R_F values of 0·2, 0·5, 0·6 and 0·7 were also observed (Table 4). The peaks having R_F values of 0·5, 0·6 and 0·7 were approximately equal in

Table 4. *Radioactivity in peaks separated by thin-layer chromatography with solvent system A (benzene–methanol–glacial acetic acid, 90:5:5, by vol.*)*

Sample	R_F...	Radioactivity† in peaks migrating to				
		0	0·2‡	0·5	0·6	0·7§
Rat bile‖, untreated		84·8		6·4	3·9	4·9
Rat bile‖, acid-treated		69·1	1·4	3·9	5·3	20·2
Rat urine		7·2	14·6	64·9	13·4	
Dog bile, untreated		63·7		4·2		32·1
Dog bile, acid-treated		5·6	1·9		12·0	80·0
Dog urine		15·2		71·5	13·4	
Pig bile, untreated		93·0		7·0		
Pig urine		14·8	5·3	29·0	24·0	26·9

*The R_F of DEHP in this system was 0·9.

†Values are percentages of total radioactivity found on thin-layer chromatograms assayed by scraping 5-mm sections from origin to fronts and counting with the liquid scintillation counter.

‡Region to which *o*-phthalic acid migrated.

§Region to which mono-(2-ethylhexyl) phthalate migrated.

‖Bile samples were obtained from rats by bile-duct cannulation during a 4-hr period immediately after [^{14}C]DEHP administration.

size and accounted for approximately 80% of the total radioactivity on the plate. The substance that migrated to R_F 0·7 coincided with authentic MEHP, and the substance that migrated to R_F 0·2 was indistinguishable from *o*-phthalic acid.

Aqueous–organic partition of excreta

Aqueous–organic partition was performed on the excreta of dogs and pigs to determine the nature of the excreted radioactivity. In the urine of dogs, an average of 90·5% of the radioactivity was found in the aqueous phase, and in the urine of pigs, 88·4% was found in the aqueous phase. Examination of faecal matter by this technique indicated that 37·4% was found in the aqueous phase (62·6% being organic-extractable) in dogs and 67·1% was found in the aqueous phase (32·9% organic-extractable) in pigs.

DISCUSSION

After oral administration of 50 mg [^{14}C]DEHP/kg to male Sprague–Dawley rats, 37·5 and 53·2% of the administered radioactivity was excreted in the urine and faeces, respectively, in 4 days. These results are in reasonable agreement with those of Daniel & Bratt (1974), even though the dosage levels differed (2·9 mg/kg *v.* 50 mg/kg). Schulz & Rubin (1973) administered an oral dose of 200 mg/kg to rats and found 40·2% of the dose in the urine, 22·9% in the faeces and 17·4% in the gastro-intestinal tract in 24 hr; we found 27·3% of the dose in the urine, 56·7% in the faeces and 6·0% in the gastro-intestinal tract 24 hr after a 50 mg/kg dose. Approximately 70% of the radioactivity in the tissues and excreta of the rat was found in the aqueous phase upon aqueous-organic partition (Schulz & Rubin, 1973).

Time-course analysis of the blood of both dogs and pigs showed an initial rapid decline followed by a low, but sustained, level of radioactivity. The initial blood elimination half-life of 1·4 hr in dogs and 3·0 hr in pigs indicated rapid elimination of [^{14}C]DEHP from the blood, either by distribution to tissues or by excretion. The sustained level of radioactivity in blood after the initial rapid decline appears to indicate either a release of ^{14}C material from storage depot(s) or enterohepatic recycling or a combination of both phenomena. Radioactivity from [^{14}C]DEHP was present in the gastro-intestinal tract and bile of both dogs and pigs over a period of several days; this observation may indicate that [^{14}C]DEHP or its metabolites were secreted in the bile and underwent enterohepatic recycling. TLC analysis of the bile samples obtained at 4 hr from rats, dogs and pigs showed that virtually no unchanged DEHP was present. The metabolites of DEHP therefore appear to undergo enterohepatic recycling. Dog bile contained 6–58 times the level of radioactivity found in pig bile, indicating that biliary secretion of DEHP metabolites occurred to a greater extent in the dog than in the pig. The fact that the faecal route of excretion was major (75·1%) in dogs and minor (25·7%) in pigs lends support to this conclusion.

A small amount of the radioactivity from [^{14}C]DEHP was distributed into muscle and fat. In the dog and pig, the level of radioactivity in both fat and muscle did not decrease as rapidly as it did in tissues such as liver or lung. The amounts of body fat and muscle in both dogs and pigs were estimated from published values, and no allowance was made for slight individual variations in morphology. This variation is probably the cause of fluctuations in our values, particularly in the pig (Table 3).

Schulz & Rubin (1973) reported that virtually no unchanged DEHP was excreted in the urine of rats; the aqueous–organic partition and TLC studies of urine from our experimental animals confirm this observation. Our TLC studies on whole urine and solvent-fractionated samples showed the presence of as many as four metabolites of [^{14}C]DEHP in the urine of rats, three metabolites in the urine of dogs and five in the urine of pigs.

The faecal route of excretion was predominant in the dog (75·1% in 4 days), was substantial in the rat (53·2% in 4 days) and accounted for one quarter (25·7% in 4 days) of the dose administered to pigs. Because extensive molecular transformations of compounds by intestinal microbes are likely to occur in the large intestine, no attempt was made to characterize the radioactive products found in faecal matter other than by aqueous–organic partition. Relatively nonpolar substances were present in dog faeces and more polar substances were present in pig faeces. However, the extent of metabolism of [^{14}C]DEHP cannot be estimated solely on the basis of the results of aqueous–organic partition of faeces.

Since faecal excretion was the major excretory route in the dog and because bile samples contained a substantial level of radioactivity, attempts were made to characterize the metabolites of [^{14}C]DEHP present in dog bile. TLC analysis and extraction and solvent fractionation indicated that the major radioactive constituent of bile was an extremely polar substance which remained at the origin when the plate was developed in solvent A. Because mildly acidic conditions (0·1 M-acetate buffer, pH 5·0) readily destroyed the polar substance and resulted in the formation of substances slightly more polar than MEHP, the major radioactive constituents of dog bile may be ester-type conjugates, possibly glucuronic esters, which would be acid-labile. The aglycones, because of their relative polarity compared with MEHP, may contain oxidized alkyl chains, as Albro *et al.* (1973) reported in studies of DEHP metabolism in rats.

TLC examination of urine and bile from dogs indicated that the metabolites of DEHP present in urine were different from those in bile. The bile contained substantial amounts of a substance that had the same R_F as MEHP in solvent system A. Apparently this substance was then further metabolized to a more polar (R_F 0·5) substance which was excreted in the urine. Chu *et al.* (1978) reported the presence of chain-oxidized metabolites in urine after administration of MEHP to rats, and postulated that MEHP was an intermediate in the metabolism of DEHP. Our results in the dog and rat appear to be consistent with this postulate.

The major metabolite of DEHP found in pig bile appeared to be a highly polar substance, and a small amount of substance migrated to R_F 0·5 in solvent system A. Since pig bile did not contain a great amount of the administered ^{14}C dose, further workup of these samples was not performed. In the urine from

pigs, at least five different substances were observed. One of these substances migrated with MEHP, and the substances with R_F values of 0·5 and 0·6 appeared to be the same substances found in urine from rats and dogs. A small amount of a substance migrating like *o*-phthalic acid (R_F 0·2) was also observed, and some of the radioactivity remained at the origin.

For comparison purposes, rat bile, collected by bile-duct cannulation, was examined by TLC. After development of the plates in solvent system A, the major radioactive constituent of rat bile remained at the origin (R_F 0); mild acid treatment of the bile sample resulted in a slight decrease in the peak at R_F 0 and a quadrupling of the small peak that migrated to R_F 0·7 (Table 4). These results appear to indicate the presence of acid-labile conjugates of metabolite(s) of DEHP in rat bile.

TLC of rat urine indicated that the urinary metabolites of DEHP were different from those in bile. Although it is possible that the substances with R_F values of 0·5 and 0·6 may be common to both urine and bile, rat urine contained no substance with an R_F of 0·7. In rat urine, a substance that migrated like *o*-phthalic acid (R_F 0·2) was present and a minor substance remained at the origin (Table 4).

Our TLC results indicate that [^{14}C]DEHP has a similar metabolic fate in rats and dogs, except that no substance migrating with *o*-phthalic acid was found in the urine of dogs. The miniature pig formed more metabolites from [^{14}C]DEHP than did the rat or dog, and excreted a substance in the urine that migrated with MEHP in solvent system A.

The metabolites in the bile of rats and dogs appeared to be somewhat similar and the presence of an acid-labile constituent was indicated. However, dog bile appeared to contain more of a constituent that under mildly acidic conditions formed a substance that migrated with MEHP in solvent system A, and rat bile appeared to contain a compound that did not readily undergo hydrolysis under these conditions.

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THE VARIABILITY OF DIETARY FIBRE IN LABORATORY ANIMAL DIETS AND ITS RELEVANCE TO THE CONTROL OF EXPERIMENTAL CONDITIONS

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Abstract—The importance of dietary fibre in laboratory animal nutrition lies in its capacity to affect experimental results, especially those in toxicology. Thirty-five diets for mice, rats, guinea-pigs, rabbits, primates, dogs and cats have been analysed for dietary fibre constituents, namely pectin, hemicellulose, cellulose and lignin. Both the total fibre content of the diets, and the proportions of its constituents are very variable and are related to the formula of the diet. The possible consequences for research are discussed.

INTRODUCTION

Probably the single most important factor that has held back our understanding of dietary fibre is the adherence to the nineteenth century crude fibre method of analysis. The indigestible components of diets are so diverse as to make a single definition almost impossible. Generally these substances originate in the plant cell wall where they are often physically interlinked. Analysis of the various fractions is laborious, and complete resolution requires a week's work (Southgate, 1969). Simpler methods involve selective dissolution of the chemically distinct forms (Robertson, 1978). These methods are considerably more useful physiologically than the crude fibre method, but manufacturers of laboratory animal diets still quote only crude fibre analysis in their advertising literature. Because of the extra expense of fibre-free feeds, most manufacturers would prefer to produce diets with as much fibre as might be tolerated by the animals (Lang & Briggs, 1976). Dietary fibre levels are also important to the staff of animal houses who have to clear up faecal waste (Lang & Briggs, 1976). The quantity of faeces produced was found to increase eightfold when dietary fibre was changed from 0 to 25% (Forsythe, Chenoweth & Bennink, 1978).

Recent developments in laboratory animal science have tended to stress the importance of standardizing experimental animals, the strain used, their freedom from pathogens, and their housing in unstressful environments. It is recognized that diet too must be controlled because alterations in nutrient composition are potent sources of biochemical variation that cause anomalous experimental results (Clarke, Coates, Eva, Ford, Milner, O'Donoghue, Scott & Ward, 1977; ILAR, 1978). However, dietary fibre is not considered a nutrient because it cannot be digested by endogenous intestinal enzymes. The ILAR (1978) regards cellulose as a relatively inert ingredient which can be used to make up the difference in diet composition when the concentration of one nutrient is changed experimentally. This is common practice in nutritional work (Lang & Briggs, 1976), but close examination of the literature reveals many instances when anomalous results cannot be explained on the basis of known nutrients. Furthermore, it seems that cellulose and other non-nutritive fillers are not physiologically inert and require stringent control as do nutrients themselves.

In man dietary fibre is important because of its effects on colon function which are related to disease (Mitchell & Eastwood, 1976). However, in the diets of laboratory animals, the importance of fibre lies in its capacity to affect experimental results. The possibility that dietary fibre levels might affect the results of toxicity tests is particularly disturbing since these results are used to assess the safety of various chemicals for use in the human environment. Ershoff (1974) has reviewed the results of several of his own experiments and considers them to demonstrate that dietary fibre affected the toxicity of test chemicals. Among the toxins which are less active in diets containing fibre, Ershoff lists glucoascorbic acid, polyoxyethylene sorbitan monostearate, chlorazanil hydrochloride, sodium cyclamate, and amaranth. Since then, tartrazine and Sunset Yellow FCF have been added to this list (Ershoff, 1977). Kritchevsky (1977) found evidence in the literature to suggest that 2-acetylaminofluorene, 2,5-di-*tert*-butyl hydroquinone and cadmium chloride could also be added to this list.

Mylroie, Moore, Olayi & Anderson (1978) found a much lower toxicity of lead in a stock diet than in a semisynthetic diet, while tumour incidence was similarly much lower in rats given 7,12-dimethyl benz[*a*]-anthracene with a stock diet than with a semisynthetic diet (Carroll, 1975). Clinton, Edes, Truex & Visek (1979) showed that bran prevented exposure of the intestine to benzo[*a*]pyrene as measured by the dose-dependent induction of intestinal aryl hydrocarbon hydroxylase. Although cellulose alone may be partially effective in lowering toxicity, the greatest effect has been found when natural mixtures of dietary fibre are used.

The influence of dietary fibre is not restricted to orally administered toxins. Subcutaneously adminis-

tered dimethylhydrazine caused less colon cancer when rats were fed bran (Fleiszer, Murray, MacFarlane & Brown, 1978), but this experiment has been severely criticized by Cruse, Lewin & Clark (1978). Gardner & Heading (1979) found that ip injections of 5-flourouracil caused proportionately much less decrease in mucosal peptidase activity in rats fed stock diet than in those fed one of several synthetic diets. Although most results tend to indicate the protective influence of dietary fibre, this is not invariably the case. When Asp, Bauer, Dahlqvist, Fredlund & Oste (1978) fed pectin to rats, sc injections of dimethylhydrazine caused a significantly greater number of colorectal and ear duct tumours compared to rats fed a fibre-free diet, while wheat bran did not affect tumour development. When indomethacin was injected sc, it produced intestinal ulceration only when dietary fibre was present in the diet (Drees, Robbins & Crago, 1974).

In the present study a selection of commercial laboratory animal diets have been analysed for dietary fibre components. The results show which fractions are most variable and the importance of this information is discussed.

EXPERIMENTAL

Samples of approximately 100 g diet were powdered and analysed for pectin, hemicellulose, cellulose and lignin.

Duplicate analyses were made for pectin in 1 g samples of diet, one with and one without 20 mg added pectin (8% methoxy-containing ester of polygalacturonic acid from Sigma Chemical Co., St. Louis, MO, USA). Although pectin hydrolysis may not be complete, this standard addition to the diet allows the pectin in the diet to be estimated. Pectin was extracted with three 25 ml aliquots of 0·5% ammonium oxalate at 85°C (Weihe & Phillips, 1947). The supernatant containing pectin was hydrolysed with 10 ml concentrated sulphuric acid under reflux for 1 hr. This was made up to 100 ml, and 0·4 ml of this was used for colorimetric determination of galacturonic acid by the carbazole reaction, as described by Dekker & Richards (1972). According to McComb & McCready (1952), there is negligible interference from other substances likely to be present in the pectin extract.

Hemicellulose, cellulose and lignin were determined by the detergent methods of Robertson (1978). Terry & Outen (1973) have noticed that, when either maize or barley starch is present in the diet, the neutral detergent method does not remove all the starch, therefore predigestion of duplicate 0·05-g samples with amyloglucosidase was carried out as described by Macrae & Armstrong (1968). The sample was then refluxed gently for 1 hr with neutral detergent solution, filtered through a sintered glass Gooch crucible under minimal vacuum, rinsed, washed twice with acetone and dried in an oven at 102°C. The ashing step was not performed for each residue, but several ashings were performed on the combined residues to obtain average values. The same fraction was subtracted from each determination to yield neutral detergent residue.

The acid detergent residue was determined on duplicate 0·5-g samples using a different solution, but by a similar procedure to that for neutral detergent residue, without the predigestion of starch. After drying and weighing, the filtered acid detergent residues were subjected to oxidation of lignin by permanganate solution, while still in their crucibles. After drying and weighing, the delignified residues remaining in the crucibles were treated with 72% sulphuric acid, washed and dried to yield cellulose. Hemicellulose was calculated as the difference between the neutral and acid detergent residues. Total dietary fibre was calculated as the sum of the separate fractions.

Standard deviations for each method were calculated by one-way analysis of variance (Armitage, 1971).

RESULTS

The precision of the analytical methods has been expressed in terms of their standard deviations. The pectin determination had a standard deviation of 0·10 as a percentage of the sample, which is equivalent to a coefficient of variation of 10% of the mean pectin for all samples. For the neutral detergent residue the figures were 1·1 and 6%, for the acid detergent residue 0·32 and 4%, for cellulose 0·45 and 8%, and for lignin 0·15 and 8%. These errors were partly reduced by taking means of duplicate assays. Table 1 shows that the methods used gave good agreement between diets of the same formula, but produced by different manu-

Table 1. *Dietary fibre analyses for diets of published formulae produced by various manufacturers*

Formula	Manufacturer	Total dietary fibre (g/kg dry wt)	Dietary fibre constituent (% of total)			
			Pectin	Hemicellulose	Cellulose	Lignin
FD1	Labsure	293	6·2	47·1	37·9	8·9
	BP	343	7·8	51·6	31·9	8·7
RGP	Labsure	275	3·9	52·7	31·6	11·8
	Dixons	312	4·6	57·0	31·3	7·1
41B	Labsure	164	2·9	51·6	33·0	12·5
	Dixons	186	3·5	57·9	28·0	10·6
SG1	Grain Harvesters	349	4·3	58·2	28·6	8·9
	Dixons	314	5·1	55·3	31·7	7·9

Table 2. *Levels of fibre-containing constituents in some laboratory animal diets of published formulae*

Dietary constituent	Level of constituent (g/kg) in diet of formula			
	FDI	RGP	41B	SGI
Barley	255	400	—	—
Wheat	—	—	450	—
Oats	100	125	400	120
Wheat feed	150	150	—	180
Bran	—	—	—	400
Soya extract	—	100	—	—
Linseed extract	100	—	—	—
Grass	300	150	—	200

facturers. Table 2 shows the type of ingredients, and variabilities of published formulas. After all the dietary fibre extractions had been made, there remained an average of 0·6% of the sample as a residue, the chemical nature of which is unknown. It may be a mixture of cutin, other complex polymers and silica.

Analyses of the dietary fibre are presented in Table 3 in order of dietary fibre content for each species. Average total dietary fibre contents of mouse, rat, primate and dog diets were similar, but those of guinea-pigs and rabbits were roughly twice as high. Although they are quite well-correlated ($r = 0·71$), crude fibre analyses provided by the manufacturers represent only one third of the total dietary fibre.

Overall the dietary fibre content of diets ranged from 6% for a cat diet to 35% for a rabbit diet. The proportion of pectin in the dietary fibre varied from 3 to 18%, the cellulose from 18 to 45% and the lignin from 6 to 21%. There was also considerable variation between the diets for a given species, especially in the mouse, rat and cat diets. The dietary fibre in mouse and rat diets ranged from 8 to 22%. The two cat diets analysed were very different, one being lower in fibre than any of the other diets, while the other was similar to an average mouse and rat diet. The proportions of each type of fibre were also variable especially among the mouse and rat diets, with more than a threefold range of pectin and twofold ranges for cellulose and lignin.

Table 3. *Dietary fibre content and breakdown of laboratory animal diets*

Species	Diet	Total dietary fibre (g/kg dry wt)	Dietary fibre constituent (% of total)			
			Pectin	Hemicellulose	Cellulose	Lignin
Guinea-pig	BP FDI	343	7·8	51·6	31·9	8·7
	Dixons RGP	312	4·6	57·0	31·3	7·1
	Labsure FDI	293	6·2	47·1	37·9	8·9
	Labsure RGP	275	3·9	52·7	31·6	11·8
Mouse & rat	Spratts maintenance	224	2·6	64·1	23·3	10·0
	Grain Harvesters 465	193	3·6	61·5	27·4	7·5
	BP No. 3	190	6·7	61·2	24·0	8·1
	Dixons 41B	186	3·5	57·9	28·0	10·6
	Labsure PRD	174	4·1	53·6	30·6	11·7
	Grain Harvesters 475	168	3·1	64·7	18·3	13·9
	Labsure 41B	164	2·9	51·6	33·0	12·5
	BP No. 1	163	7·3	65·0	19·8	7·9
	Spratts breeding	162	3·3	58·9	26·1	11·7
	Labsure CRM	159	4·7	59·4	27·6	8·3
	Labsure PMD	143	4·1	43·5	41·1	11·3
	Oxoid pasteurized	127	5·4	48·8	31·5	14·3
	Dixons FFG (M)	120	7·2	65·3	19·6	7·9
	Spratts lab 1	83	11·4	36·7	36·3	15·6
Cat	Carnation Go-cat	139	7·1	39·3	32·7	20·9
	Quaker Felix Crunch	56	18·1	35·2	28·6	18·1
Dog	BP dog diet	191	5·3	65·9	19·7	9·1
	Grain Harvesters vitaflake	141	4·9	62·3	23·8	9·0
	Spratts dog diet no. 1	139	10·9	53·5	23·9	11·7
Primate	Labsure CPD2	171	4·2	64·3	22·3	9·2
	BP Mazuri diet	149	7·3	61·0	21·8	9·9
	Grain Harvesters primate diet	148	4·7	58·1	24·1	13·1
	BP old world monkey diet	146	10·8	56·9	24·3	8·0
	BP marmoset diet	143	7·6	50·0	27·5	14·9
	Dixons FPI	135	6·8	61·5	26·2	5·5
Rabbit	Grain Harvesters SGI	349	4·3	58·2	28·6	8·9
	Labsure CRB	315	2·5	45·1	43·7	10·7
	Dixons SGI	314	5·1	55·3	31·7	7·9
	Spratts special rabbit diet	310	3·5	43·3	40·9	12·3
	Labsure R14	297	2·9	53·9	32·3	10·9
	Grain Harvesters 474	280	5·1	39·4	45·5	10·0

DISCUSSION

There is vast scope for further research on the subject of dietary fibre, both in human nutrition and in laboratory animal nutrition. If animal experiments are intended to be relevant to man, the diet of the animals studied should be similar to that of man or if this is impossible, the experimentalist should be very careful in the interpretation of his results, especially when comparing results obtained with stock diets and those with semisynthetic diets. At present it is impossible to mimic the dietary fibre content of stock diets by additions to semisynthetic diets, and attempts to do this with cellulose powder must be interpreted for what they are, additions of only one constituent of dietary fibre, and that too from wood or cotton rather than food. Because the diet of western man tends to be low in dietary fibre, it could probably be compared to a semisynthetic diet, while stock diets resemble some unrefined African human diets.

The results obtained in the present series of analyses demonstrate that stock diets themselves are standard neither in their total dietary fibre content nor in the proportions of its constituents. Although there is considerable argument about the methods that should be used for dietary fibre analyses, those used here have been established for some time among animal nutritionists, and they are probably adequate for comparative purposes. No simple method is yet available to give a complete breakdown of the dietary fibre as it exists in the food. Each component is itself made up of many interlinked compounds, and the combined properties will depend on the way they are linked and the extent of their methylation. The user of experimental animals needs some comparative index of the functions of dietary fibre, but this is not yet available. Therefore, the present results may be used as an indication of the range of different types of fibre that are likely to be found in different animal diets.

To predict the effect that dietary fibre may have on individual experiments, it is necessary to understand the physiological actions of dietary fibre components in the intestine. Some of the effects of dietary fibre can be explained on the basis of its enhancement of bacterial fermentation, but the extent of fermentation is very dependent on species since some animals have little or no adaptation to a plant diet while others are adapted to a totally vegetarian diet. Hungate (1976) has given a good account of the various fermentation systems in different animals. Ruminants and the rather rare animals with in-line pre-acid fermentation are not dealt with in this paper. Most laboratory animals have post-acid fermentation in the caecum or large intestine, while a few carnivores that are used may have little capacity for fermentation (Banta, Clemens, Krinsky & Sheffy, 1979). However, the effects of dietary fibre in the small intestine are similar for both types of animal.

Effects of dietary fibre in the small intestine

It is generally held that dietary fibre is unaffected by endogenous digestive enzymes in the small intestine, and therefore its effects are due to its adsorptive, cation exchange, and swelling properties. However, Viola, Zimmerman & Mokady (1970) have reviewed research which indicates that some alkaline degradation of pectin to uronic acids may occur in the small intestine of rats, and these substances may have some metabolic effects. Dietary fibre is capable of swelling in the presence of water and therefore increases intestinal volume (McConnell, Eastwood & Mitchell, 1974). There is considerable variation among different sources of dietary fibre with regard to the extent to which they swell in the intestine. It seems likely that the bulking effect may help to cause the faster transit times through the gut that are associated with increased dietary fibre intakes (Mitchell & Eastwood, 1976).

Different kinds of dietary fibre have variable cation exchange capabilities. By reducing the availability of sodium in the intestine, it has been suggested that dietary fibre may reduce the rate of water absorption, and hence increase intestinal volume (Mitchell & Eastwood, 1976). Another important consequence of its cation exchange capabilities is that calcium, zinc and iron deficiencies could result from excessive dietary fibre (Cummings, 1978; Reinhold, Ismail-Beigi & Faradji, 1975). The calcium-chelating properties of 10% dietary pectin have been shown to reduce calcium absorption considerably (Viola *et al.* 1970). It appears to be the uronic acid portion of dietary fibre (i.e. pectin and hemicellulose) that binds calcium (James, Branch & Southgate, 1978). The cation exchange capacities of dietary fibre can also explain its effect on lead and cadmium toxicities. However, another substance in unrefined diets, phytate is a potent cation-binding agent and can also affect absorption of cations (Davies, 1978). Adsorption of many substances to dietary fibre has been shown to occur. The adsorbent properties of cellulose are routinely used in chromatography (Lang & Briggs, 1976). It is also known that lignin is an active adsorbent, and it tends to bind to the hydrophobic part of molecules (Eastwood & Hamilton, 1968). For example, adsorption of bile salts is greatest when acidic groups are unionized, and bile salts with a lower number of hydroxyl groups are more easily bound because of their greater hydrophobic characteristics. Some inconsistent experimental results might be partly explicable by the action of saponins, which may bind bile salts to their hydrophobic group, while themselves being bound to the non-lignin fibres *via* their hydrophilic group (Oakenfull & Fenwick, 1978). Other experiments may have been affected by differences in fibre particle sizes because the smaller the particle, the greater the surface area to volume ratio and the greater the binding capacity (Burczak & Kellogg, 1979). Bile salt adsorption may be relevant to the effects of dietary fibre on lipid metabolism (Kritchevsky, 1978). The binding of nitrosamines to dietary fibre has been shown to occur (Wishnok & Richardson, 1979), and this observation may have considerable toxicological importance.

Effects of dietary fibre in the caecum and large intestine

The second site of action of dietary fibre is in the caecum and large intestine. Lignin is practically unaffected by microbial fermentation and is not a carbohydrate (Gordon, 1978). Therefore the basic structure of some plant material may still be microscopically little changed after passage through the intestine (George, Reeves & Harbers, 1978). Pectin is almost

completely fermented by rats (Cullen & Oace, 1978), but Gilmore (1966) found that low-methyl-pectin was less readily fermented than a high-methyl type. Hemicellulose is more than twice as readily fermented as cellulose in rat diets (Key, Van Soest & Young, 1970). There is considerable argument about whether mice and rats obtain useful amounts of energy from dietary fibre fermentation (Hove & King, 1979; Viola *et al.* 1970, Yang, Manoharan & Young, 1969), but it seems more or less certain that rabbits and guinea-pigs do (Hagen & Robinson, 1953; Hoover & Heitmann, 1972). The volatile fatty acids also stimulate peristalsis and might therefore speed up transit through the intestine (Hellendoorn, 1973). They may also attract extra water into the intestine by osmosis (Forsythe *et al.* 1978). Similarly, the gas produced during fermentation increases faecal volume and reduces transit time (Hellendoorn, 1973). Approximately 0·1 to 0·2% of dietary energy was lost by rats as methane daily, and five times as much by guinea-pigs (Rodkey, Collison & O'Neal, 1972).

Bacteria in the large intestine use the energy derived from fermentation to grow and multiply. Bacterial mass largely accounts for the increase in faecal bulk when extra fermentable dietary fibre is fed in the diet (Hellendoorn, 1978). The bacteria use nitrogen that would otherwise have been reabsorbed (Stephen & Cummings, 1979), and this may explain the observation of Meyer (1958) that dietary cellulose increases protein requirement.

There is also very considerable scope for interaction between microbial metabolism and dietary fibre. For example, Chadwick, Copeland & Chadwick (1978) suggested that the protective effect of dietary fibre against lindane toxicity might be due to enhanced bacterial metabolism of lindane.

Conclusion

The differences in dietary fibre content and constituents observed in these diets are important for all common laboratory animals, and should be controlled as far as possible in experiments, especially those in toxicology. It should also be borne in mind that animals kept on edible bedding obtain some dietary fibre from it. Therefore if control of dietary fibre is the criterion, bedding should not be used.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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AMYL FORMATE

Synonym: Pentyl formate.
Structure: Mixed *n*-amyl and isoamyl formates:

$$CH_3 \cdot [CH_2]_4 \cdot OOC \cdot H \text{ and } CH_3 \cdot CH(CH_3) \cdot [CH_2]_2 \cdot OOC \cdot H.$$

Description and physical properties: EOA Spec. no. 185.
Occurrence: Reported to be a constituent of *Pyrus malus* (*Fenaroli's Handbook of Flavor Ingredients*, 1975) and to be found in apples, tomatoes, whisky and honey (CIVO–TNO, 1977).
Preparation: By the esterification of primary amyl alcohols and formic acid.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·0025	0·05
Maximum	0·06	0·06	0·02	0·3

Analytical data: Gas chromatogram, RIFM no. 77–21; infra-red curve, RIFM no. 77–21.

Status

Amyl formate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was listed by the Council of Europe (1974) with an ADI of 5 mg/kg. CAS Registry No. 638–49–3.

Biological data†

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

Irritation. Amyl formate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was slightly irritating (Moreno, 1977). Tested at 3% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 28 volunteers. The material (RIFM no. 77–21) was tested at a concentration of 3% in petrolatum and produced no sensitization reactions (Epstein, 1977).

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†Literature searched from 1962 through 1979.

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ANISYL PHENYLACETATE

Synonyms: *p*-Methoxybenzyl phenylacetate; phenylacetic acid, *p*-methoxybenzyl ester.
Structure: $CH_3 \cdot O \cdot C_6H_4 \cdot CH_2 \cdot OOC \cdot CH_2 \cdot C_6H_5$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From anisyl alcohol and phenylacetic acid by direct esterification with a catalyst under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·2
Maximum	0·1	0·01	0·03	0·7

Analytical data: Gas chromatogram, RIFM no. 77–27.

Status

Anisyl phenylacetate is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974), at a level of 5 ppm, in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. CAS Registry No. 102–17–0.

Biological data*

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

Irritation. Anisyl phenylacetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Moreno, 1977). Tested at 12% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–27) was tested at a concentration of 12% in petrolatum and produced no sensitization reactions (Kligman, 1977).

Metabolism. In substituted anisoles with a carboxyl group or a potential carboxyl group attached to the aromatic ring, the ether link is relatively stable (Williams, 1959).

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BACCARTOL

Structure: A complex material.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By reaction of citronella oil with acetone under condensation conditions.
Uses: Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·007	0·001	0·004	0·12
Maximum	0·04	0·004	0·02	0·4

Analytical data: Gas chromatogram, RIFM no. 77–31; infra-red curve, RIFM no. 77–31.

Status

Baccartol is not included in the listings of the FDA, FEMA (1965–1979) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972). CAS Registry No. 68916–62–1.

Biological data*

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

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

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–31) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1977).

References

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*Literature searched from 1962 through 1979.

n-BUTYL CINNAMATE

Synonyms: *n*-Butyl phenylacrylate; cinnamic acid, butyl ester; 2-propenoic acid, 3-phenyl-, butyl ester.
Structure: $C_6H_5 \cdot CH{:}CH \cdot COO \cdot [CH_2]_3 \cdot CH_3$.
Description and physical properties: A colourless oily liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of *n*-butanol with cinnamic acid under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·0025	0·08
Maximum	0·06	0·006	0·02	0·4

Analytical data: Gas chromatogram, RIFM no. 76–321; infra-red curve, RIFM no. 76–321.

Status

Butyl cinnamate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was listed by the Council of Europe (1974) with an ADI of 1·25 mg/kg. CAS Registry No. 538–65–8.

Biological data*

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

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

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 76–321) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1977).

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Moreno, O. M. (1977). Report to RIFM, 7 October.

*Literature searched from 1962 through 1979.

BUTYL CINNAMIC ALDEHYDE

Synonyms: α-Butyl cinnamaldehyde; α-*n*-butyl-β-phenylacrolein; 2-(phenylmethylene)hexanal.
Structure: $C_6H_5 \cdot CH{:}C([CH_2]_3 \cdot CH_3) \cdot CHO$.
Description and physical properties: A pale-yellowish oily liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By condensation of *n*-hexaldehyde with benzaldehyde (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 5000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·4
Maximum	0·3	0·03	0·1	0·8

Analytical data: Infra-red curve, RIFM no. 77–51.

Status

Butyl cinnamic aldehyde was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) at a level of 1 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. CAS Registry No. 7492–44–6.

Biological data*

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

Irritation. Butyl cinnamic aldehyde applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately to severely irritating (Moreno, 1977). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–51) was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1977).

References

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Moreno, O. M. (1977). Report to RIFM, 30 September.

*Literature searched from 1962 through 1979.

n-BUTYL ISOVALERATE

Synonyms: *n*-Butyl isopentanoate; butyl isovalerianate; isovaleric acid, butyl ester; butanoic acid, 3-methyl-, butyl ester; butyl 3-methylbutyrate.
Structure: $CH_3 \cdot [CH_2]_3 \cdot OOC \cdot CH_2 \cdot CH(CH_3) \cdot CH_3$.
Description and physical properties: A colourless liquid.
Occurrence: Reported to be found in the oil from leaves of *Eriostemon coxii* and *Phebalium dentatum* (*Fenaroli's Handbook of Flavor Ingredients*, 1975), and in bananas and pears (CIVO–TNO, 1977).
Preparation: By direct esterification of *n*-butanol with isovaleric acid under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·0005	0·0025	0·04
Maximum	0·03	0·003	0·01	0·15

Analytical data: Gas chromatogram, RIFM no. 77–54; infra-red curve, RIFM no. 77–54.

Status

Butyl isovalerate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974), at a level of 50 ppm, in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. CAS Registry No. 109–19–3.

Biological data*

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1978). The oral LD_{50} of butyl isovalerate in rabbits was reported by Munch (1972) to be 52 mmol/kg (8·2 g/kg). The ND_{50} (narcotic dose, defined as the quantity producing stupor and loss of voluntary movements in half of a test group) was 38 mmol/kg (6 g/kg) in rabbits, with doses above this causing disappearance of corneal reflexes, nystagmus, dyspnoea and bradycardia (Munch, 1972).

The toxicity of butyl isovalerate in comparison with that of homologous esters has been related to the degree of saponification and the length of the acid portion of the molecule in studies involving ip administration of the LD_{50} to rats (Selisko, Ackermann & Kupke, 1962).

Irritation. Butyl isovalerate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was slightly irritating (Moreno, 1978). Tested at 1% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1978).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 28 volunteers. The material (RIFM no. 77–54) was tested at a concentration of 1% in petrolatum and produced no sensitization reactions (Epstein, 1978).

Nutrition. Only 10% of the energy from butyl isovalerate was available when the ester was fed to chicks at a level of 5% in the diet (Yoshida, Morimoto & Oda, 1970).

Metabolism. Butyl isovalerate was detected in cows' milk 2 hr after administration of 1 g, dissolved in acetone and fed through the rumen with an odourless diet (Honkanen, Karvonen & Virtanen, 1964).

Pharmacology. Butyl isovalerate, among other components, was used in a synthetic fermented egg product that acted as a coyote (*Canis latrans*) attractant and deer (*Odocoileus hemionus columbianus*) repellent (Bullard, Shumake, Campbell & Turkowski, 1978).

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Agrochemicals

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**Chemical Abstracts* section heading.

n-BUTYL PROPIONATE

Synonyms: Propionic acid, butyl ester; propanoic acid, butyl ester.
Structure: $CH_3 \cdot [CH_2]_3 \cdot OOC \cdot CH_2 \cdot CH_3$.
Description and physical properties: *Merck Index* (1976).
Occurrence: Reported to be found in several natural products (*Fenaroli's Handbook of Flavor Ingredients*, 1975), including apples, bananas, raspberries and blackberries (CIVO-TNO, 1977).
Preparation: By direct esterification of *n*-butanol with propionic acid (or propionic anhydride) under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·005	0·04
Maximum	0·05	0·005	0·02	0·2

Analytical data: Gas chromatogram, RIFM no. 77–57; infra-red curve, RIFM no. 77–57.

Status

n-Butyl propionate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was listed by the Council of Europe (1974) with an ADI of 1 mg/kg. CAS Registry No. 590–01–2.

Biological data*

Acute toxicity. The acute oral LD_{50} in rats was reported as approximately 5 g/kg and the acute dermal LD_{50} in rabbits as >5 g/kg (Moreno, 1978). The toxicity of butyl propionate in comparison with that of homologous esters has been related to the degree of saponification and the length of the acid portion of the molecule in studies involving ip administration of the LD_{50} to rats (Selisko, Ackermann & Kupke, 1962).

Irritation. *n*-Butyl propionate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1978). Tested at 2% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1978).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 27 volunteers. The material (RIFM no. 77–57) was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Epstein, 1978).

Nutrition. The energy from butyl propionate was 65% available when the ester was fed to chicks at a level of 5% in the diet (Yoshida, Morimoto & Oda, 1970).

Pharmacology. Butyl propionate, among other compounds, was used in a synthetic fermented egg product that acted as a coyote (*Canis latrans*) attractant and deer (*Odocoileus hemionus columbianus*) repellent (Bullard, Shumake, Campbell & Turkowski, 1978).

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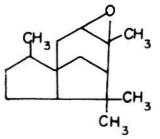
Nandi, B. (1978). Glucosamine analysis of fungus-infected wheat as a method to determine the effect of antifungal compounds in grain preservation. *Cereal Chem.* **55**, 121; cited from *Chem. Abstr.* 1978. **89**, 4732.

*Arrangement is based on section headings from *Chemical Abstracts*.

CEDR-8-ENE EPOXIDE

Synonym: Andrane.
Structure:



Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From epoxidation of cedrene, followed by distillation.
Uses: In public use since the early 1960s.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·3
Maximum	0·15	0·015	0·05	1·2

Analytical data: Gas chromatogram, RIFM no. 77–26; infra-red curve, RIFM no. 77–26.

Status

Cedr-8-ene epoxide is not included in the listings of the FDA, FEMA (1965–1979) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data*

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1978).

Irritation. Cedr-8-ene epoxide applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was slightly to moderately irritating (Moreno, 1978). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1978).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 28 volunteers. The material (RIFM no. 77–26) was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Epstein, 1978).

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Epstein, W. L. (1978). Report to RIFM, 27 Febuary.

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Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

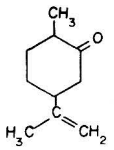
Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Moreno, O. M. (1978). Report to RIFM, 1 February.

*Literature searched from 1962 through 1979.

DIHYDROCARVONE

Synonym: *p*-Menth-8-en-2-one; 2-methyl-5-(1-methylethenyl)cyclohexanone.
Structure:



Description and physical properties: A colourless to pale-yellowish liquid.
Occurrence: Reported to be found in the essential oils of caraway, anethum, *Mentha spicata* var. *longifolia* and angelica seeds (*Fenaroli's Handbook of Flavor Ingredients*, 1975) and in celery, mint and pepper (CIVO–TNO, 1977).
Preparation: From dihydrocarveol by oxidation (Arctander, 1969).
Uses: In public use since the 1960s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	1·5
Maximum	0·3	0·03	0·1	2·0

Analytical data: Gas chromatogram, RIFM no. 77–121; infra-red curve, RIFM no. 77–121.

Status

Dihydrocarvone was given GRAS status by FEMA (1978), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) in the list of artificial flavouring substances not fully evaluated. CAS Registry No. 7764–50–3.

Biological data*

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977). The sc LD_{50} for dihydrocarvone administered in sesame oil to male mice was 2900 mg/kg; signs of extreme excitement, convulsions and respiratory depression were noted (Wenzel & Rose, 1957).

Irritation. Dihydrocarvone applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1977). Tested at 20% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 28 volunteers. The material (RIFM no. 77–121) was tested at a concentration of 20% in petrolatum and produced no sensitization reactions (Epstein, 1977).

Pharmacology. Hexobarbital sleeping time was decreased in comparison with control values when 500 mg dihydrocarvone/kg was administered sc in sesame oil to mice (Wenzel & Ross, 1957). The same dose administered ip to rats was not active in reversing pentobarbital-depressed respiration, but an increase in running activity compared with control values followed ip administration of 50–400 mg/kg in sesame oil to rats (Wenzel & Ross, 1957).

Enantiomeric *cis*- and *trans*-dihydrocarvones produced different odour perceptions in man (Russell & Hills, 1971).

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*Literature searched from 1962 through 1979.

Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.
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*Arrangement is based on section headings from *Chemical Abstracts*.

DIMETHYLBENZYLCARBINYL BUTYRATE

Synonyms: α,α-Dimethylphenylethyl butyrate; butyric acid, α,α-dimethylphenethyl ester; benzyldimethylcarbinyl *n*-butyrate.
Structure: $C_6H_5 \cdot CH_2 \cdot C(CH_3)_2 \cdot OOC \cdot [CH_2]_2 \cdot CH_3$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of dimethylbenzylcarbinol with *n*-butyric acid, using a catalyst and preferably azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·15
Maximum	0·15	0·015	0·04	1·0

Analytical data: Gas chromatogram, RIFM no. 77–129; infra-red curve, RIFM no. 77–129.

Status

Dimethylbenzylcarbinyl butyrate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. CAS Registry No. 10094–34–5.

Biological data*

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

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

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–129) was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1977).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, no. 992. S. Arctander, Montclair, NJ.

Council of Europe (1974) Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List 2, no. 2084. p. 300. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2394. *Fd Technol., Champaign* **19** (2), part 2, 155.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. (1977). Report to RIFM, 15 September.

Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Moreno, O. M. (1977). Report to RIFM, 18 October.

*Literature searched from 1962 through 1979.

DIMETHYLBENZYLCARBINYL PROPIONATE

Synonyms: Benzene ethanol, α,α-dimethyl-, propanoate; benzylisopropyl propionate; phenethyl alcohol, α,α-dimethyl-, propionate.
Structure: $C_6H_5 \cdot CH_2 \cdot C(CH_3)_2 \cdot OOC \cdot CH_2 \cdot CH_3$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of dimethylbenzylcarbinol with propionic acid or propionic anhydride (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·15
Maximum	0·1	0·01	0·04	1·0

Analytical data: Gas chromatogram, RIFM no. 77–130; infra-red curve, RIFM no. 77–130.

Status

Dimethylbenzylcarbinyl propionate is not included in the listings of the FDA, FEMA (1965–1979) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972). CAS Registry No. 67785-77-7.

Biological data*

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

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

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 28 volunteers. The material (RIFM no. 77–130) was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Epstein, 1977).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol 1, no. 995. S. Arctander, Montclair, NJ.

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Epstein, W. L. (1977). Report to RIFM, 7 October.

FEMA (1965–1979). Recent progress in the consideration of flavoring ingredients under the food additives amendment. GRAS substances. A series of ten articles: *Fd Technol., Champaign* **19** (2), part 2, 155 (1965); **24** (5), 25 (1970); **26** (5), 35 (1972); **27** (1), 64 (1973); **27** (11), 50 (1973); **28** (9), 76, (1974); **29** (8), 70 (1975); **31** (1), 65 (1977); **32** (2), 60 (1978); **33** (7), 65 (1979).

Food Chemicals Codex (1972). 2nd Ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406, Washington, DC.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

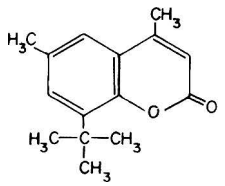
Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Moreno, O. M. (1977). Report to RIFM, 18 October.

*Literature searched from 1962 through 1979.

4,6-DIMETHYL-8-*tert*-BUTYLCOUMARIN

Structure:



Occurrence: Has apparently not been reported to occur in nature.
Preparation: By the condensation of *o*-*tert*-butyl-*p*-cresol with ethyl acetoacetate in the presence of a strong acid, such as sulphuric acid.
Uses: Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·15
Maximum	0·1	0·01	0·03	0·8

Status

4,6-Dimethyl-8-*tert*-butylcoumarin is not included in the listings of the FDA, FEMA (1965–1979) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972). CAS Registry No. 17874-34-9.

Biological data*

Acute toxicity. Both the acute oral LD_{50} in mice and the acute dermal LD_{50} in guinea-pigs exceeded 5 g/kg (Moreno, 1978).

Irritation. 4,6-Dimethyl-8-*tert*-butylcoumarin applied full strength to intact or abraded guinea-pig skin for 24 hr under occlusion was slightly irritating (Moreno, 1978). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1978).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 78–83) was tested at a concentration of 8% in petrolatum and produced one sensitization reaction (Kligman, 1978).

Phototoxicity. 4,6-Dimethyl-8-*tert*-butylcoumarin at a concentration of 5% in hydrophilic ointment did not produce any phototoxic effects on human subjects (Kaidbey, 1979).

Photoallergenicity. 4,6-Dimethyl-8-*tert*-butylcoumarin produced photoallergenic effects on seven of 25 human subjects when tested at a concentration of 1% in hydrophilic ointment by the photomaximization test (Kligman & Kaidbey, 1978). Human subjects who were photoallergic to 6-methylcoumarin or to 7-methoxycoumarin did not cross-react to 4,6-dimethyl-8-*tert*-butylcoumarin (Kaidbey, 1979).

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Council of Europe (1974). Natural Flavouring Substances. Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

FEMA (1965–1979). Recent progress in the consideration of flavoring ingredients under the food additives amendment. GRAS substances. A series of ten articles: *Fd Technol., Champaign* **19** (2), part 2, 155 (1965); **24** (5), 25 (1970); **26** (5), 35 (1972); **27** (1), 64 (1973); **27** (11), 50 (1973); **28** (9), 76, (1974); **29** (8), 70 (1975); **31** (1), 65 (1977); **32** (2), 60 (1978); **33** (7), 65 (1979).

Food Chemicals Codex (1972). 2nd Ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406, Washington, DC.

Kaidbey, K. H. (1979). Report to RIFM, 18 January and 8 February.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm* **47**, 393.

Kligman, A. M. (1978). Report to RIFM, 24 October.

Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Kligman, A. M. & Kaidbey, K. H. (1978). Report to RIFM, 2 October.

Moreno, O. M. (1978). Report to RIFM, 4 October.

*Literature searched from 1962 through 1979.

3,7-DIMETHYLOCTANYL ACETATE

Synonyms: 1-Octanol, 3,7-dimethyl-, acetate; tetrahydrogeranyl acetate; dihydrocitronellyl acetate.
Structure: $CH_3 \cdot COO \cdot [CH_2]_2 \cdot CH(CH_3) \cdot [CH_2]_3 \cdot CH(CH_3) \cdot CH_3$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 3000 lb/yr.

Concentration in final product (%):

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

Analytical data: Gas chromatogram, RIFM no. 77–137; infra-red curve, RIFM no. 77–137.

Status

The Council of Europe (1974) included 3,7-dimethyloctanyl acetate in the list of artificial flavouring substances not fully evaluated. CAS Registry No. 20780–49–8.

Biological data*

Acute toxicity. The acute oral LD_{50} in rats was reported as >5 g/kg and the acute dermal LD_{50} in rabbits as approximately 5 g/kg (Moreno, 1977).

Irritation. 3,7-Dimethyloctanyl acetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was slightly to moderately irritating (Moreno, 1977). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 28 volunteers. The material (RIFM no. 77–137) was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Epstein, 1977).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List 3 (Section A), no. 4225, p. 395. Strasbourg.

Epstein, W. L. (1977). Report to RIFM, 7 October.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Moreno, O. M. (1977). Report to RIFM, 19 October.

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Boelens, H., Ter Heide, R. & Rijkens, F. (1968). Chemical structure and odor. VI. Geraniol derivatives. *Am. Perfumer Cosm.* **83**, 27 & 32; cited from *Chem. Abstr.* 1969, **70**, 50420.

*Literature searched from 1962 through 1979.
†*Chemical Abstracts* section heading.

3,7-DIMETHYLOCTANYL BUTYRATE

Synonyms: Butanoic acid, 3,7-dimethyloctyl ester; tetrahydrogeranyl butyrate.
Structure: $CH_3 \cdot [CH_2]_2 \cdot COO \cdot [CH_2]_2 \cdot CH(CH_3) \cdot [CH_2]_3 \cdot CH(CH_3) \cdot CH_3$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

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

Status

Dimethyloctanyl butyrate is not included in the listings of the FDA, FEMA (1965–1979) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972). CAS Registry No. 67874-80-0.

Biological data*

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

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

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 24 volunteers. The material (RIFM no. 77–134) was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Epstein, 1977).

References

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DIMETHYL SUCCINATE

Synonyms: Methyl succinate; succinic acid, dimethyl ester; butanedioic acid, dimethyl ester; dimethyl butanedioate.
Structure: $CH_3 \cdot OOC \cdot CH_2 \cdot CH_2 \cdot COO \cdot CH_3$.
Description and physical properties: *Merck Index* (1976).
Occurrence: Reported to be found in filbert nuts (*Fenaroli's Handbook of Flavor Ingredients*, 1975).
Preparation: Directly from methanol by esterification with succinic acid under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·08
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 77-141; infra-red curve, RIFM no. 77-141.

Status

Dimethyl succinate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974), at a level of 100 ppm, in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. CAS Registry No. 106-65-0.

Biological data*

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1978).

Irritation. Dimethyl succinate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1978). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77-141) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1977).

Nutrition. The energy in dimethyl succinate was not available when the ester was fed to eight chicks at a level of 5% in the diet (Yoshida, Morimoto & Oda, 1970).

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DIMYRCETOL

Structure: A mixture of dihydromyrcenol and dihydromyrcenyl formate:
$CH_3 \cdot CH_2 \cdot C(:CH_2) \cdot [CH_2]_3 \cdot C(CH_3)_2 \cdot OH$ and $CH_3 \cdot CH_2 \cdot C(:CH_2) \cdot [CH_2]_3 \cdot C(CH_3)_2 \cdot OOC \cdot H$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Uses: In public use since the 1960s. Use in fragrances in the USA amounts to approximately 3000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·3
Maximum	0·2	0·02	0·08	1·5

Analytical data: Gas chromatogram, RIFM no. 77–144; infra-red curve, RIFM no. 77–144.

Status

Dimyrcetol is not included in the listings of the FDA, FEMA (1965–1979) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 4·1 g/kg (3·5–4·8 g/kg) and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1977).

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

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–144) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1977).

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p-ETHOXYBENZALDEHYDE

Synonym: 4-Ethoxybenzaldehyde.
Structure: $CH_3 \cdot CH_2 \cdot O \cdot C_6H_4 \cdot CHO$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By ethylation of *p*-hydroxybenzaldehyde (Arctander, 1969).
Uses: In public use before the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·2
Maximum	0·3	0·03	0·1	0·8

Analytical data: Gas chromatogram, RIFM no. 77-155; infra-red curve, RIFM no. 77-155.

Status

p-Ethoxybenzaldehyde was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974), at a level of 0·5 ppm, in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. CAS Registry No. 10031-82-0.

Biological data*

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·1 g/kg (1·8–2·5 g/kg) and the acute dermal LD_{50} in rabbits as >5 g/kg (Moreno, 1977).

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

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77-155) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1977).

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Review Section

Genetic Toxicology

This review considers the issues addressed by the Scientific Committee of the Food Safety Council related to genetic toxicology as a part of their four year task to study the body of knowledge regarding food safety testing and to recommend appropriate criteria for the assessment of risk of food ingredients. The Committee concludes its evaluation of existing tests with recommendations. However, as noted, any comments on the state of the art in the field of mutagenic testing is necessarily incomplete and provisional considering the dynamic state of evolution of knowledge in the genetic sciences.*

Introduction

Diseases of genetic origin may affect human health in a variety of ways from covert existence to total debility and death. About 10% of all human disease may have a genetic component and this burden may be considered as falling into three categories.

The best defined category of diseases with a genetic component are those due to chromosomal aberrations detectable by cytological techniques. Examples are mongolism (Down's syndrome), Klinefelter's syndrome, Turner's syndrome and possibly some forms of mental retardation. Virtually all cases are due to new mutations which have arisen in the maternal or paternal germ cells. The underlying genetic mechanisms are either chromosomal nondisjunction in meiosis, or chromosomal translocations between nonhomologous chromosomes at some point during gametogenesis, or mixed events e.g. nondisjunction combined with small translocations. Other events such as chromosomal deletions, duplications and inversions have also been observed. Nondisjunction plays however the major role in causing aneuploidy in man. Autosomal aneuploidies are, in general, associated with more severe phenotypic effects than sex chromosomal aneuploidies.

The disease or morphological expression of any chromosomal mutation in a species depends, in part, on the particular genes affected in the mutant chromosome and takes a form characteristic only for that species. It is possible to investigate changes in the rate of chromosomal mutations in man and intact mammals by comparison of the observed rate against natural background mutation rates.

The next best known category of diseases of genetic origin includes those due to single dominant genes expressed, in the majority of cases, as overt structural anomalies or malformations. About 100 such dominant gene mutations are known in man and these include hereditary neoplasms e.g. bilateral retinoblastoma.

While some disease burden is owing to new mutations, the rest is inherited from past generations. The hereditary mechanism involved is the occurrence of a mutant gene in an individual which is subsequently expressed as an overt mutant trait. Fifty percent of the offspring from such an individual will inherit the gene and express that trait in the absence of any modifying characteristics.

The third category of genetically determined diseases encompasses disorders due to recessive genes. There are over 1000 known human diseases exhibiting the inheritance characteristic of recessive gene mutations e.g. sickle cell anaemia, cystic fibrosis, Tay-Sachs disease, phenylketonuria and some other inborn errors of metabolism. The hereditary mechanism involved requires for expression a homozygous individual to whom a maternal and paternal

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mutant gene has been transmitted simultaneously. Heterozygous individuals possessing only one mutant gene are apparently normal carriers, except when small deletions involve several recessive genes, resulting in limited expression. Heterozygous carriers greatly outnumber homozygous diseased individuals.

Recessive gene mutations remain unexpressed and undetected, being passed on through many generations, until from a single mating homozygosity results. The human population already carries a significant number of such genes and new mutations at the site of the unaffected gene complement occur with a definite probability. It is not possible to estimate this proportion of homozygotes contributed by new mutations.

The genetic mechanisms underlying both dominant and recessive gene mutations are either point mutations or small interstitial deletions. Because the number of genes in mammals (markers) which can be studied with present day methods is limited, large numbers of animals are needed to detect significant changes in mutation frequency at marker loci. Studies on human mutation rates for newly formed recessive genes are not available.

Many common human disorders e.g. diabetes, epilepsy, schizophrenia, arthritis and hypertension are presumed to have a genetic component, the nature of which is poorly understood. These disorders are presumed to be transmitted as multiple gene disorders with a familial tendency. Whether the natural mutation rate for these disorders is affected by mutagenic agents is unknown, hence no conclusions can be drawn regarding the relevance of current mutagenicity tests for this group of diseases.

The occurrence of true genetic disease in the human population is difficult to estimate and even more so the contribution of chemical agents to the human genetic disease burden. Some defective genes or chromosomes manifest themselves phenotypically with birth defects or are lost as spontaneous abortions, others find phenotypic expression later in life as dominant or recessive autosomal or sex linked disorders. Even mere heterozygosity for a deleterious recessive mutation may incur disadvantages with regard to viability despite the phenotypic dominance of the unmutated gene (Legator, 1977).

There are sound theoretical reasons for concern over the possibility that chemical mutagens may threaten the integrity of the human genome (the totality of genes and chromosomes) and thus affect future generations of man. There can be no reasonable doubt of the occurrence of mutations. For all practical purposes these mutations can either be considered neutral or deleterious yet man has survived the presence of environmental mutagens through thousands of generations. The greatly increased exposure of the human population to man-made chemicals as a result of the industrial revolution requires that the impact of these chemicals on the health and economy of the human community be set against the undoubted benefits of their introduction. The potential genetic hazards to man from some chemicals presently in wide use may be the production of genetic lesions in somatic cells, possibly leading to cancer, or the induction in germ cells of transmissible gene mutations and chromosome alterations in structure and number, which may affect future generations. The possibility of increasing the incidence of any genetic disease in future generations is a matter of vital concern, particularly the possibility that inherited genetic changes may increase the susceptibility to carcinogenic influences. This has been shown for certain repair-deficiency mutations, even in heterozygous conditions (Swift et al., 1976).

Although a doubling of the total genetic load arising from normal background mutations may not be tolerable, small increases in the genetic burden relative to the background mutation rate may be unavoidable and thereby become acceptable. The selection pressure against adverse human mutations is less in modern society, so that many mutants with genetic non-fitness survive, and thus have an impact on the gene pool by increasingly polluting it with consequent greater genetic risks. An

example is the survival of homozygotes for retinoblastoma to reproductive age following enucleation treatment. Similarly, phenylketonurics and other carriers of inborn metabolic errors survive today on adequate therapy. Nevertheless, the risk from the induction of new dominant mutations is smaller than that from gene pollution by surviving mutants or by newly induced recessive and undetected mutations. The latter do not become apparent nor are they being selected against, being only mildly deleterious, until their sheer number leads to homozygosity expressed in wholly unpredictable phenotypism. Moreover, any increases in mutation rate over the past 50 years or so, during which the enhanced exposure to synthetic chemicals has occurred, could not contribute a significant number of fresh mutants to the already existing recessive gene mutation pool derived from natural background mutations of all previous generations. These cryptic new mutations thus remain a legacy to future generations. However, if, in theory, exposure to chemicals will produce new chromosomal or severe dominant gene mutations, then such mutations are more easily detected.

It should also be remembered, that genes vary in their capacity to tolerate injury. For example, the gene for the crucial enzyme involved in homogentisic acid metabolism, is known to contain about 1000 nucleotides. It can tolerate several changes, so that only 10% of mutations in this gene become expressed as alkaptonuria, the other 90% remaining neutral and lead to polymorphism. Similarly the globin gene can tolerate up to 90% of base substitutions leaving a fit gene while only 10% of the mutations result in sickle cell anemia. On the other hand about 95% of additions, deletions and frameshifts result in non-functional detrimental genes (Flamm, 1977).

Objectives of Mutagenicity Testing

The first objective of testing a chemical in mutagenicity assays is the establishment of its capability, if any, to induce heritable genetic effects in man. The identification of this property of a substance is not possible by direct human investigations. Hence non-human and even non-mammalian test systems have been developed to resolve this question. Since gametogenesis involves both mitotic and meiotic processes, assessment of mutagenicity should ideally be done on cells which undergo meiosis i.e. germinal cells. However, cells other than germinal cells have proved to be very useful indicator systems for particular genetic alterations.

The second objective is the categorization of the risk of mutagenicity to man. Since many compounds with potential genetic activity cannot be eliminated from use or from the environment, some risk evaluation is necessary for the prediction of acceptable or tolerable exposure levels. It has been suggested that mutagenic risk may be quantified in relation to background mutation rates. This approach has been used for both ionizing radiation and chemical mutagens, similar to the assessment of the risk from chemical carcinogens. It is questionable if any meaningful numerical extrapolation can be made from animal data in the present state of the art. Based on results from a battery of tests, the chemical can be categorized as to high, moderate, or low mutagenic potential. This approach is described in the section on recommendations.

The third objective is the use of the information derived from mutagenicity testing for predicting other toxicological properties, particularly the potential for inducing cancer. The assessment of the usefulness of short-term mutagenicity tests for predicting carcinogenic activity requires considerable efforts to establish satisfactory correlations and to reduce to a minimum the possibilities of obtaining either false positive or false negative results. In addition, it must be emphasized that mutagenicity tests alone cannot define a substance as a carcinogen. Only appropriate carcinogenicity bioassays are capable of furnishing acceptable evidence of such a property. The correlation of mutagenic activity with teratogenic potential is far less securely established, the induction of

somatic mutations in the developing embryo having been observed to produce only very few terata. There are also theoretical grounds for suspecting a role for somatic mutations in the process of aging.

The mutagenic "fingerprint" or spectrum of mutagenic activity of a chemical may indicate that it may have undergone modification as a result of industrial processing or passage through biological systems.

Indicator systems for detecting mutagenic activity in body fluids, cytogenetics, sperm abnormalities, and alkylation of macromolecules, may provide useful tools for monitoring relevant population groups for exposure to biologically hazardous compounds, thus achieving an earlier warning than is provided by traditional epidemiological endpoints. Similarly, food may be screened for significant interactions as a result of technological processing or compositional modification. Mutagenic assay systems may be practical tools for identifying those fractions of food likely to contain active compounds or for investigating body fluids and tissue extracts for the presence of mutagenically active metabolites.

The results of mutagenicity tests may be used for decision making regarding the acceptability of exposure of man to substances anywhere in the environment, the suitability of a chemical for premarketing development or its use as a therapeutic agent, or for priority ranking vis-a-vis chronic toxicological investigations. Certain facts concerning these short-term tests must, however, be remembered. Many of these procedures have been developed only recently and require great skill and expertise on the part of the investigator in order to yield reliable and reproducible results. These conditions are often satisfied in the hands of the originators of the tests or in those of a few other skilled and highly experienced experts. As the circle of users in testing laboratories expands, there is a real danger that the reliability of the results will be progressively reduced through lack of intimate familiarity with the essential minutiae of the test procedures. This includes not only their complexities but also their potential for producing artifacts or other misleading results.

This comment is not meant to impugn the excellence of the tests nor the competence of the investigators. It is intended as an explicit recognition of the numerous complex and hitherto unresolved issues inherent in every one of these tests. Accordingly, before action is taken on the basis of the results of such short-term tests, it is essential to review scrupulously the conduct of the tests and to confirm the results, if necessary. Uncertainty still exists as to extent of the negative evidence needed to counterbalance a positive finding in a mutagenicity assay but completely negative findings carry reasonable assurance of the absence of mutagenic potential.

Scientific Background

It is now established that the genetic material of all living organisms is the DNA except for some RNA viruses. The genetic information itself is encoded in the sequence of base pairs in the DNA, such that 3 bases specify one amino acid. The code is universal, the same triplet corresponding to the same amino acid whatever the source of DNA. The double helical structure of the DNA, as discovered by Wilkins, Watson and Crick, explains the replication process in chemical terms. The organisational levels of DNA extend from base pairs through triplet codons to unique recognition sequences. Each level encodes specific information and ensures the faithful inheritance of this information.

A mutation may be regarded as a disruption of the code at any level of DNA organisation, thereby changing the genetic information either in germ cells or in somatic cells. The change consists of two kinds, gene mutation by chemical alterations of an individual gene to give a mutated allele or chromosome alterations by the gain or loss of a section with consequent rearrangement. A mutagenic substance increases the observed natural frequency of chromosome alterations as well as gene mutations. Only mutations, that are pro-

duced in the germ line or germ cells of individuals prior to or during the reproductive period, are capable of transmission to later generations where their effects may become apparent; however, changes in the genetic materials of somatic cells and tissues cannot be ignored because of their possible relationship to carcinogenicity and teratogenicity.

Chemically induced *gene mutations* fall into several categories:

1. Point mutations affecting one or more nucleotides or chemical alterations in one or a few bases out of 10^3-10^5 bases in the DNA portion of individual genes result in alteration in the informational content of genes. The changed information is then translated into mistakes in the protein controlled by the gene which may become apparent, for example, as a loss of enzyme function or loss of structural function. Examples are: a) Frameshifts in which a small number of base pairs are added or deleted as a result of agents intercalating between bases by covalent binding. As a consequence, the codon triplet reading frame is shifted forward (deletion) or backward (addition) by one or more bases except when 3 consecutive bases or multiples of 3 consecutive bases are affected. As a consequence the cell synthesizes greatly altered nonfunctional proteins unless the frameshift occurs near the end of the gene. In that case most of the activity of the protein may be preserved. Similarly a second frameshift in the opposite sense to the first lesion may restore the frame with comparatively little change in the protein structure. b) Base-pair substitutions in which one nucleotide is replaced by another. Theoretically it is possible to have transitions where one purine base is replaced by another and transversions where a purine is replaced by a pyrimidine. The result may be a change in the codon of an amino acid so that another amino acid will be substituted on translation. Alternatively the new codon becomes a chain terminator if the new base triplet represents a nonsense code. Some of these base-pair substitutions have no effect but many are deleterious if they result in chain termination or abolishing of protein function. Base substitutions may also involve replacement of a nucleotide by a base analog, when at subsequent replication mispairing occurs. Alternatively, binding of a mutagen with a base in the karyotic or extrakaryotic DNA, e.g., O^6-G alkylation, will produce an abnormal base which mispairs at replication unless the lesion is repaired. SOS repair could however lead to mutation before replication. In the absence of repair a heritable permanent change has occurred in the daughter cell genome. This event is found in organs with specific repair deficiency which then become the target site for the reactive chemical. c) Intercalation without covalent binding may also change the DNA without causing frameshifts, but nevertheless leads to a mutagenic response.

2. Changes in groups of genes involving the introduction of new genes from isolated exogenous DNA, e.g. virus nucleic acid, into chromosomes. Alternatively, small deletions may involve only part of a gene or several genes without any recognizable structural changes in the chromosome.

3. Recombinations, which are a reciprocal exchange of information between two homologous DNA molecules or chromosomes or between non-homologous sites of any two chromosomes. Recombination leads, in general, to a combination of genes on one single chromosome that is different from that present in each of the parental chromosomes.

4. Loss of gene function may occur as a result of alteration of the regulator functions.

Chemically induced *structural chromosome aberrations* affect the structure of the chromosome and occur as a result of misrepair of breaks leading to deletions, duplications or translocations. Large deletions affecting part of a gene or several genes result in loss of the associated protein fraction. *Numerical aberrations* in the whole chromosome set arise from nondisjunction with loss or gain of an extra chromosome. These changes result from alterations in many bases. The structural or numerical alteration in the chromosomal apparatus of the cell can be regarded as a further event occurring only if a sufficient concentration of

mutagen is present to proceed from a simple alteration of the genome to a macroscopic genetic lesion (breaks) or rearrangement of genetic information (translocations) or to a shift in numerical patterns (non-disjunction). There is a significant number of chemicals which disrupt the chromosomal organization of the cell inducing numerical or structural chromosome abnormalities without producing cell mutation.

Covalent binding of the chemical to DNA may result in a variety of DNA damage. The adduct may be stable but alkalilabile, at a site insensitive to endonuclease attack. This lesion then persists unrepaired, either without overt functional or phenotypic effect or as a mutation after replication. The adduct may be a stable alkylated base, alkylation having occurred at a functional group such as $-NH_2$ or -OH, e.g. O^6-alkylate guanine, which persists unrepaired or is converted by N-glycosidase into an apurinic or apyrimidinic site sensitive to endonuclease attack. Eventually single strand breaks occur either directly as a result of base alkylation or of endonuclease activity. Alternatively the sites of loss of alkylated base may lead to template inactivation with subsequent cytotoxicity and cell death. If the adduct is an unstable alkylated base, alkylation having occurred in the ring, e.g. N^7-alkylated guanine, it is lost by spontaneous hydrolysis creating thereby apurinic or apyrimidinic sites.

Demonstration of interaction of a parent compound, or a metabolite, with DNA *in vitro* is not conclusive evidence for mutagenic action but accords the compound a high priority for biological testing. Whole-body radioautography gives rapid information on the distribution of compounds and metabolites, in particular indicating penetration to the germinal tissues. Covalent binding to cellular or organelle constituents may give additional information on reactivity with macromolecules.

Following the reaction of a chemical with DNA, the latter changes into a mutant form. Initial lesions in DNA such as pyrimidine dimers, single strand breaks, base alkylation or cross-linking of two strands may be lethal, remain permanently or be repaired by several processes. Error-free repair may restore a normal genome while error-prone repair may lead to a mutational lesion or result in a dying cell. The enzymes involved in the repair processes may be altered in their activities. Excessive numbers of DNA lesions may overload normal repair processes and may induce the SOS (error-prone) repair system.

DNA repair probably involves several distinct mechanisms in mammalian cells. One process is excision (cut and patch) or pre-replication repair. The chemical lesion, recognized by a complex system, is removed by two types of processes, a) nucleotide excision, b) base removal.

In nucleotide excision either a long patch of about 100 nucleotides (even if the initial damage involves only 1-3 nucleotides) or a short patch of 1-10 nucleotides is excised. The first step is incision in the vicinity of the lesion by an endonuclease, the resulting single strand gap varying in size for different damaging agents. The excision of the stretch of DNA containing the region of damage is followed by copy synthesis of a patch using the opposite intact strand as template. The final step is rejoining the patch and strand by a ligase to reconstitute the original strand thus restoring the fragmented DNA to its full length.

In base removal or zeropatch repair the first step is either spontaneous or DNA-glycosidase mediated removal of the faulty base resulting in an apurinic or apyrimidinic site. This is then repaired by endonuclease incision and removal of the damaged nucleotides followed by synthesis of a patch and rejoining of the strand.

Excision repair is normally error-free and occurs also in human cells. It is not blocked by hydroxyurea which stops semiconservative DNA replication as a result of normal mitosis.

The other process involves bypassing of the lesion in the newly synthesized daughter DNA, thus leaving a gap, which is sealed in a way at present unknown in mammalian cells. In bacteria a DNA segment is inserted by recombination into the new daughter DNA. Hence it is known as post-replication (bypass) repair and may be more error-prone. As a var-

iant the postreplication gap may be filled by DNA synthetized *de novo*. It thus corrects errors copied by replication.

A third process which is recombination repair is established in bacteria but has not been demonstrated conclusively in mammalian cells. There may be other variants e.g. non-dimer repairs.

Mutations can also occur in DNA regions which do not code for proteins e.g. the operator region or in the mRNA (messenger) and tRNA (transfer). The role of mistranslation from RNA has not been explored in depth. All these mutations may affect protein synthesis or transcription yet the cell may survive. Point mutations are more easily studied in haploid organisms since no masking by the normal gene in the homologous chromosome of the diploid organism can occur.

In *E. coli* there is good experimental evidence that UV induces pyrimidine dimers as the DNA lesion causing either lethality or mutation. uvrA mutants deficient in excision repair and the double mutant recA, uvrA are 60 to 3000 times more sensitive to dimer formation than wild type *E. coli*. However, as both repair and replication go on at the same time, some mutations always result even in repair competent strains.

Metabolic activation of the inactive form of the mutagen (premutagen) to the active ultimate mutagen is probably necessary in many cases but direct mutagens, active without metabolic intervention, also exist. The detection of such reactive mutagens is a complex problem. Their presence may not be recognized, if these reactive metabolites fail to reach their cellular targets. *In vitro* assays using metabolic activation systems may miss the *in vivo* effects of diet, enzyme induction, buccal and intestinal bacterial flora, stress or hormonal status. Metabolic activation may be the result of non-enzymic reactions or of activation by tissue enzymes or by the intestinal flora of the host.

The importance of the relationship of mutagenesis to germ cell stage is illustrated by the observation that both the absolute and relative frequencies of the various genetic endpoints depend on the germ-cell stage exposed to the mutagen. In the sexually mature male mammal all germ cell stages e.g. stem cells and all gamete maturation stages as well as mitotic and meiotic cell divisions are present simultaneously. In the mature female no mitotic or early meiotic prophase cells exist, all oocytes are in an arrested stage, the first meiotic division occurring just before ovulation. To sample the whole range of stages it is possible a) to expose the animal once or for a short time and make matings subsequently during the interval between the days for the latest and earliest germ cell stage to become a mature gamete; b) to expose the animal repeatedly for the difference in days required for the latest and earliest germ cell stage to mature and to perform single mating at the time when the latest germ cells have matured after the end of the exposure. A positive result must be followed by the single exposure procedure to determine the most sensitive germ cell stage.

From the point of view of human genetic risk the most important germ cell stages are the arrested oocyte and the spermatogonial stem cell.

Little information is available on additive, synergistic or inhibitory effects in chemical mutagenesis, nor on the effect of impurities in the test substance on the response of assay systems.

Utility of Existing Test Systems

It has recently been recognized that many man-made chemicals (drugs, pesticides, cosmetics, air and water pollutants, food additives, household and industrial chemicals) in our environment have potent mutagenic activity on a wide range of organisms. Thus have developed the new research fields of environmental mutagenesis and genetic toxicology in public health. Further, it was also recognized that carcinogenic and mutagenic potential

overlap and that many compounds capable of producing malignant transformation are also able to effect genetic changes. It remains to be resolved how many mutagens are also carcinogens, but there is a clear need to evaluate the effect on the human genome of exposure of the individual to a genotoxic agent, to determine the likelihood of successful transmission of any damage and to assess the possible effect on future generations (De Serres, 1976). To attack this problem, research has been directed to the development of mass screening techniques using rapid, efficient and inexpensive assay systems.

The overall potential for inducing different types of genetic damage uniquely characterizes a substance in terms of its mutagenic activity in the same way as its metabolic fingerprint and its individual spectrum of other toxicological properties. Because mutations involve distinct genetic events which may result from different molecular mechanisms, an ideal comprehensive assay system should measure accurately all these events. However, most presently employed assays detect only a few endpoints, so that a chemical with a unique action may be positively mutagenic in one system and negative in others. It is therefore necessary to be aware of the limitations of the capabilities of any assay system by testing it against a large number of mutagens and non-mutagens of differing chemical structures. Moreover, chemical mutagens produce highly specific types of genetic damage unlike radiation e.g. only non-disjunction but no gene mutations of chromosome aberrations; only point mutations etc. Because of the specificity of mutagenic activity frameshift mutagens cannot revert basepair substitutions and vice versa. Similarly reversion tests will have a negative outcome if the incorrect translation or transversion is produced. Since complete reversion assay screens covering all possible substitutions and frameshifts are not available, it is best to use the forward mutation of the whole gene. This assumes that such assays are equisensitive to specific reversion assays with the added advantage of responding to a wider range of potential mutagens. From these considerations follows that a battery of tests, and not a single assay, must be used in the investigation of the mutagenic potential of a compound. General utilization of this new approach will permit the detection of mutagens and potential carcinogens in the human environment, will prescreen and select priorities for long-term carcinogenicity testing, thus rapidly evaluating numerous hitherto untested environmental chemicals in terms of their mutagenic and carcinogenic potential.

The test battery of choice, once properly validated, should permit the determination of mutational effects on the genome and the chromosomal structures in germ cells and somatic cells, and, if possible, any dose-effect relationships. The screening tests employed should analyze all possible genetic changes, be non-specific and detect genetic damage potentially relevant to man. Also needed is a knowledge of the correlation between different types of genetic changes induced by a single mutagen and the relationship of concentration in the target tissue to the type of genetic change.

Mutagenicity assays have as their endpoint the detection of some genetic damage. This damage may be a mutation at individual genetic loci, chromosomal damage e.g. deletions, chromatid and chromosomal breakage and repair, and evidence of DNA damage and repair. Some mutagenicity tests assess only the presence or absence of potential mutagenicity, others provide also quantitative data on which may be based categories of concern in respect of potential mutagenic risk for man. The latter utilize higher organisms in order to measure, without ambiguity, transmissible genetic events against a low spontaneous mutation rate for the same events. Transmitted effects in mammals can be used to study all of the main endpoints of mutagenesis, e.g. point mutations, chromosome breakage with or without rearrangement, and chromosome nondisjunction. In choosing suitable test systems consideration must be given to using tests covering, as far as possible, the types of genetic damage generally considered as causes of

human disorders, as well as incorporating mammalian metabolism. Relevant genetic information may possibly be obtained without necessarily conducting specific mutagenicity assays by using animals from acute toxicity tests for cytogenetic studies (e.g. chromosomal aberrations in somatic bone marrow cells and peripheral lymphocytes, and in spermatogonia and oocytes) and for the analysis of body fluids for the presence of mutagenic metabolites. Information about germ cell stage specificity can be obtained from animals on subchronic tests by adding the dominant lethal test.

The use of *in vitro* microsomal preparations, combined with microorganisms or other indicators as an exclusive primary screen, is an approach which has serious deficiencies. An *in vitro* microsomal activating system cannot reflect the complex metabolic processes occurring in the intact animal nor can it mirror the variability in the genotype of the activating tissue from which it is prepared. Indeed, it is not possible to devise a standard *in vitro* activation system that can be used generally for all potential mutagens and carcinogens. The fact, that many substances are either potentiated or detoxified by other routes e.g. the intestinal flora, would argue against relying entirely on the use of *in vitro* activating systems for the initial screening. It is noteworthy that those chemicals which induce non-disjunction by affecting spindle mechanisms, one of the most important and serious cytogenetic abnormalities, would be missed by bacterial studies. The use of whole mammals provides the closest practical approach to the metabolic conditions existing in man. Reports of occasional non-parallelism between lower system results and whole-mammal findings highlight the possible pitfalls, if assessments of risk to man are based only on the lower-system results. Determinations of mutagenic potential in whole mammals answer questions on mutagenesis *per se* as well as being indicative of carcinogenic potential in man.

Microbial systems utilizing genetic effects occurring in hotspots on the DNA attempt to indicate 1 hit on perhaps 20 affected genes. Mammalian cells in culture are a system which is more likely to detect active agents inducing chromosome aberrations by exposure of all chromosomal material to attack but it fails to expose the whole genome, if used as a system for mutation detection. All these systems suffer inherently from the danger that they may fail to give a positive indication for mutagenic activity unless they are capable of detecting the specific DNA alterations produced by the compound under test with or without metabolic activation by relevant tissue preparations or by the gut flora, if the latter route of exposure is important.

Point mutation induction has been studied mostly in microbial systems but can be studied also in cultured mammalian cells and *in vivo*. Chromosomal abnormalities have been studied essentially in cell cultures and *in vivo*. A somatic prescreen that probably detects both point mutations and chromosome aberrations uses whole-mammal assays.

Since transmissible point mutations and small (short multilocus) deletions and duplications are very important as genetic hazard, and also correlate with carcinogenic risk, a suitable test system must be capable of detecting these genetic changes. In submammalian systems Drosophila offers a satisfactory assay system for detecting this activity. A comparable test which can be used to classify a substance incontrovertibly as a mammalian mutagen is the specific locus test in mice. In this test morphological mutations are detected at specific loci in the progeny from matings of treated wild-type mice to mice homozygous for recessive alleles at these loci. The test is costly, laborious and allows the investigation of only few chemicals at a few concentrations. The dose-effect curves do not appear to be linear. There are however other tests e.g. the detection of skeletal mutations or the mammalian (*in vivo* somatic mutation test) spot test which can be used for classification of substances as mammalian mutagens. The fluorescent γ body test gives a good indication for such a property.

The microbial assays using *Salmonella* as indicator organism confirmed the correlation be-

tween mutagenic and carcinogenic potential in 63-92% of substances investigated (Ames et al., 1973; 1973a; Nakajima and Irvahara, 1973; McCann et al., 1975; McCann and Ames, 1976; Odashima, 1976; Rinkus and Legator, 1979a), but these figures are questionable because of non-randomness of the choice of the substances and chemical classes tested. The assay also lacks the advantages of pharmacokinetics inside and outside the target cell as found in the intact mammal. Even the combination with activating systems does not cover adequately all possibilities. Metabolic handling by other routes e.g. the gut flora or non-microsomal enzymes, is not covered, nor do the bacterial systems detect activity causing non-disjunction by colchicine-type mitotic poisons. Other microbial assay systems use different bacteria, e.g. *E. coli*, but suffer from similar drawbacks.

Chromosomal damage transmitted to the progeny is important for predicting mutagenic activity of a substance. Chromosome aberrations and breakages have been, in many instances, related to carcinogenic risk (Purchase et al., 1978). All these events are detected by many routine *in vivo* mammalian assay systems using cytogenetic analysis of appropriate cells in the bone marrow and peripheral blood, of testicular cells, the appearance of micronuclei, of dominant lethals, of heritable translocations or sex chromosome loss as end points. A positive outcome in the heritable translocation test or sex chromosome loss test points to a potential human mutagenic risk. In the former test, chromosomal rearrangements e.g. translocations between non-homologous chromosomes are detected. In the latter test, uneven distribution of chromosomes in germinal cells during meiosis is detected. However, these tests need relatively high concentrations at the target cells and carry a high risk of false negative results.

Positive results in any of the definitive mutagenic assays must be considered as relevant for man unless adequate evidence to the contrary is presented. For example, data showing that the test substance does not reach the germinal tissues or pharmacokinetic considerations which preclude the interaction of the active chemical with germinal tissue would invalidate the extrapolation of positive results to man. A negative result in an *in vivo* test in which the germ cells or the embryo are the target, may merely indicate that the target has not been reached by the chemical. The possibility of a blood—testis barrier must be considered although the spermatogonial compartment is not included in the barrier. If other endpoints in the same target are positive, then the substance is likely to have reached the testis even if one endpoint yields a negative result. Chromosomal damage from transplacental exposure must also be regarded as potentially hazardous because prenatal stem cells are at risk in these circumstances. Lesions in foetal somatic cells may lead postnatally to early cancer. Lesions in foetal germ cell lines may result in heritable abnormalities.

Chemicals are known which induce a high frequency of gene mutations in the germ line at exposure levels ineffective for cell toxicity elsewhere or for any other visible sign of damage to the organism. Traditional toxicological testing would therefore not alert to their presence nor to this hidden potential for hazard. Here sensitive mutagenic assays offer some hope of detecting these chemicals (Sobels and Vogel, 1977).

Studies on the metabolic fate of a compound and its pharmacokinetics offer opportunities for the use of bacterial mutagenesis tests and other readily applicable short-term tests. Metabolites may be too short-lived to be detectable by more traditional methods so that mutagenicity assays on body fluids (blood, saliva, milk) or on excreta (faeces, urine, bile) may be useful in the identification, separation and purification of individual metabolites (Truong and Legator, 1977). Indeed, short-term tests may be used for monitoring the exposure of human populations to mutagenic chemicals (Ames, 1974).

An alternative approach, when direct alkylation of DNA is difficult to determine, uses the alkylation of amino acid residues, specifically histidine, in haemoglobin and other tissue macromolecules. This alkylation becomes a

sensitive indicator for the electrophilic reactivity, by adduct formation, of a chemical,, when it is administered *in vivo* to experimental animal or man. Examination of haemoglobin and urine for alkylated histidine residues can be performed readily by amino acid analyzer techniques. This methodology has the added advantage of avoiding the use of radio-labelled material as well as being applicable to man (Truong and Legator, 1977). Alkylation of protein residues (e.g. histidine in haemoglobin) has been explored also by the use of isotopically labelled compounds with the object of measuring exposure and estimating the mutagenic risk engendered by such compounds (Ehrenberg, 1974; Ehrenberg et al., 1976; Osterman-Golkar et al., 1976; 1977).

This approach might be extended ultimately to estimating the excision products resulting from repair of DNA damage as has been done for methylated purines (Löfroth et al., 1974). The alkylation of certain amino acids in protein may be parallel to the rate of interaction with certain sites in the DNA. Mutagenic activity (expressed as mutation frequency), in turn, is proportional to the rate constant of the reaction with DNA for the same dose of different compounds. The integrated tissue dose over the whole lifespan of the indicator macromolecule is then calculated. It has therefore been proposed that this would allow a risk estimate. Since chemical analysis has greater resolving power than biological experimentation, it has been claimed that risk estimates are possible for low dose exposure to mutagens based on direct observation instead of extrapolation (Ehrenberg, 1977).

Categorizing of the relative mutagenic risk from various kinds of genetic damage can be done through *in vivo* procedures in whole mammal tests, particularly because the relative risk depends strongly on the exposed cell type. Of the various tests described the following are considered useful: dominant lethal studies, micronucleus tests, host-mediated assays, *in vivo* cytogenetic studies, heritable translocation tests and the mouse specific locus test.

The account of available procedures and their critical appraisal does not attempt to be complete or final, because the whole field of mutagenesis is very dynamic and constantly subject to change as a result of the rapidity with which new facts are being uncovered. Availability of assay procedures therefore does not mean that they are useful or appropriate for any given substance. As in all other branches of toxicology judgment is necessary for making an informed selection. Individual techniques tend to vary and nonreproducible and uncontrolled factors often give rise to equivocal conclusions. There is, at present, considerable debate as to the validation and standardization of many of these testing methods. For these reasons it is not possible to aim at defining the most suitable approaches for each of the objectives cited nor is it implied that every compound should be put through the whole gamut of investigations described. It should however be noted that in terms of reproducibility the frequently used tests for mutagenicity compare favorably with other toxicological procedures. In evaluating the results of these assays it must be remembered that a unique set of circumstances surrounds each chemical and that any overall assessment has therefore to proceed on a case-by-case basis and examine rationally all available facts.

Summary Tables

It is difficult at present to be precise about the relative validity and resolving power of the various tests for genetic damage that are available. To help with these assessments, Table 1 and Table 2 are provided.

Relationship to Carcinogenesis

The mechanisms by which chemicals induce cancer and the developmental stages from initial exposure to frank neoplasia are poorly understood. It has become apparent, however,

TABLE 1. TYPES OF GENETIC DAMAGE DETECTED BY CURRENTLY EMPLOYED MUTAGEN SCREENING SYSTEMS

Screening system		Type of damage detected						
		Chromosome aberrations				Gene mutations		
Category	Organism	Dominant lethality	Translocations	Deletions and duplications	Nondisjunction	Forward or reverse or both	Multiple specific locus	Induced recombination
Bacterial	Salmonella typhimurium*					+		
	Escherichia coli					+		
Fungal	Neurospora crassa*			+	+	+	+	+
	Aspergillus nidulans				+	+	+	+
	Yeast*	+			+	+	+	+
Plant	Vicia faba		+	+	+			
	Tradescantia paludosa		+	+	+	+		
Insect	Drosophila melanogaster*	+	+	+	+	+	+	+
	Habrobracon juglandis	+	+			+	+	+
	Bombyx mori	+				+	+	
Mammalian cell culture	Chinese hamster*		+	+	+	+		
	Mouse lymphoma*		+	+	+	+		
Intact mammal	Mouse*	+	+	+	+			
	Rat*	+	+	+	+			
	Man*		+	+	+			

*most frequently used organisms
Taken from Drake, 1975.

that different mechanisms may be responsible for carcinogenesis in different circumstances. Included among these circumstances are interference with hormone balance, "solid state" carcinogenesis (asbestos, plastic implants), irradiation (UV light, ionizing radiation), carcinogenesis by purely inorganic compounds unlikely to interact covalently with DNA, continued physicochemically induced tissue damage and repair (s.c. injections, partial hepatectomy, hepatotoxins), and the somatic mutation theory of cancer induction. All this mechanistic diversity is supported by experimental evidence. Therefore, tests based solely on gene mutations, chromosome aberrations or interaction with critical macromolecules as endpoints may not be suitable for detecting all types of carcinogens. In particular, substances acting by epigenetic mechanisms would give false negative results, whilst false positive results may be ascribed to inadequate animal studies, gross simplicity of the genetic test compared to *in vivo* metabolism, and failure to allow for DNA repair, immunological surveillance or early death. Tests employing alternative endpoints, e.g. cell transformation, may be needed for chemicals, which by virtue of their chemical structure or metabolism could not react covalently with DNA.

The sequence of events in carcinogenesis is a multistage process including initiation, promotion and progression. Modifying factors of the

TABLE 2. OPERATIONAL CHARACTERISTICS OF MUTAGEN SCREENING SYSTEMS.

Test system	*Time to run test*	*Relative ease of detection*	
		Gene mutations	*Chromosome aberrations*
Microorganisms with metabollic activation:			
Salmonella typhimurium	2 to 3 days	Excellent	
Escherichia coli	2 to 3 days	Excellent	
Yeasts	3 to 5 days	Good	Unknown
Neurospora crassa	1 to 3 weeks	Very good	Good
Cultured mammalian cells with metabolic activation	2 to 5 weeks	Excellent to Fair	Unknown
Host-mediated assay with:			
Microorganisms	2 to 7 days	Good	
Mammalian cells	2 to 5 weeks	Unknown	Good
Body fluid analysis	2 days	Excellent	
Plants:			
Vicia faba	3 to 8 days		Relevance unclear
Tradescantia paludosa	2 to 5 weeks	Potentially excellent	
Insects:			
Drosophila melanogaster:			
Gene mutations	2 to 7 weeks	Good to excellent	
Chromosome aberrations	2 to 7 weeks		Good to excellent
Mammals:			
Dominant lethal mutations	2 to 4 months		Unknown
Translocations	5 to 7 months		Potentially very good
Blood or bone marrow cytogenetics	1 to 5 weeks		Potentially good
Specific locus mutations	2 to 3 months	Unknown	

Modified from Drake, 1975.

carcinogenic process have been identified, e.g. initiators, promoters, diet and immunological surveillance, which act by poorly understood mechanisms. Some chemicals appear to act primarily as initiators and others primarily as promoters, although few carcinogenic chemicals appear to lack an element of both activities. Much experimental evidence suggests that initiation involves a mutational event (Dybas et al., 1977), this being the rationale for using mutagenicity assays. A hypothesis has proposed that promotion involves the conversion of recessive heterozygosity to expressed hemi- or homozygosity. If this is correct, then promoters might be detected by tests designed to uncover recessive mutations, e.g. mitotic recombination, gene conversion, partial or complete chromosome loss. It is also commonly accepted, that the transformation of a normal into a neoplastic cell involves replication of some of the events occurring during the development of cancer *in vivo*. Such an abrupt, permanent and heritable alteration in phenotype represents a somatic mutation. Phenotypic alteration can also result from stable alteration in the expression of the genome. Moreover, transformed cells may develop into neoplasms when injected into suitable hosts.

Hence strong evidence exists for a relationship between mutagenesis and carcinogenesis as induced by some chemicals. It must not be forgotten, that any evidence for a mutational event is to some extent also predictive of the potential induction of heritable genetic disease, the corresponding interpretation being important in its own right.

Because of the multifactorial causation of cancer, the mere production of an altered cell by mutation may not lead to cancer by itself. Also involved are epigenetic processes, e.g. differentiation of the altered cell and selection of those cells which are to proliferate as a result of the characteristic lack of response to growth-controlling mechanisms in the host.

Evidence in favour of a relationship between DNA modification and the development of cancer suggests a major role for point mutations in carcinogenesis, whether or not the altered gene function is accompanied by karyotype changes. It does not rule out a role for chromosomal aberrations and other mechanisms, although chromosomal aberrations do not invariably predispose to the development of cancer. There is some evidence that abnormal chromosome morphology may be the primary event leading to malignant cell transformation. It is postulated that the decrease in information with consequent imbalance of genetic material induced by various agents may be the key factor in the initiation of the neoplastic process. This imbalance may be the result of direct change in the DNA primary sequence or indirectly by affecting various cellular regulatory processes e.g. mitotic machinery, chromosomal protein, these changes being heritable. Moreover, the genetic target for transformation may be 50-100-fold larger than for gene mutations. Therefore a complex of genes and their proper functioning are involved in the process leading to transformed cells.

Chromosomal changes may be a primary event in human cancers because 1) many human cancers appear to arise from a single cell; often this cell has one or more chromosomes differing in morphology from those in the individual's non-affected normal cells; 2) persons at increased risk of cancer because of some environmental situation show increased chromosomal instability; 3) several rare genetic disorders with chromosomal instability and increased cancer risk have the ability to produce increased numbers of cells having *de novo* chromosomal rearrangements.

Evidence for this hypothesis may be obtained from non-chemical methods of disrupting chromosomal organization and determining whether this disturbance leads to transformed malignant cells. For example, asbestos and glass fibres of specific diameters have been shown to induce a range of chromosomal aberrations as karyotic changes and *in vitro* transformation.

In the present state of the art, it is necessary to include a cytogenetic endpoint in any core battery of tests for mutagenic/carcinogenic potential. Recent findings in Drosophila suggest that several chemicals induce point mutations as measured by sex linked recessive lethals and no chromosome abnormalities (dominant lethals). These conclusions were reached after evaluating chemicals that are potent clastogens in mammalians systems known. These data may indicate the differences in sensitivity of the tests used for point mutations and chromosome abnormalities in man, or an indication of significant differences in genetic lesions induced by chemicals in Drosophila as compared to mammals. These findings would not seem to diminish the importance of cytogenetic studies in mammalian systems.

Despite their great diversity in chemical structure a large number of mutagens and carcinogens appear to give rise, as a result of metabolic activation, to electrophilic derivatives (with electron-deficient sites) which then bind covalently to nucleophilic sites (electron-rich centres) in the nucleic acids and other cellular macromolecules e.g. karyotic and extrakaryotic DNA, though some mutagens, e.g. the base analogues, do not. Following the chemical reaction with DNA, the latter may change into a mutant form by a process involving the repair of genetic damage. This results

either in quasi-normal cells or amplification of the change leads to tumour induction. Hence the formal relationship between certain types of carcinogenesis and mutagenesis is supported by similarities in the covalent reactions of mutagens and carcinogens with DNA (Bartsch, 1976).

Early attempts to determine the existence of a correlation between carcinogenic and mutagenic activity failed primarily due to lack of appreciation of the necessity for metabolic activation and inadequate understanding of the molecular mechanisms of mutation. Although chemical mutagens were discovered by their action on Neurospora and Drosophila, many carcinogens were inactive mutagenically unless metabolically activated by the test organism. In fact for some carcinogens it could be shown that the level of metabolic activation by a tissue was directly related to the susceptibility of the organ as a target for tumour induction (Weekes and Brusick, 1975). The later introduction of *in vitro* activation systems, the development of microbial strains able to detect specific types of genetic alterations in DNA and the rendering of test organisms into repair-deficient strains with increased membrane permeability and enhanced responsiveness due to plasmid introduction, provided very sensitive indicator organisms.

Several surveys using these highly sensitive strains showed a high correlation of positive results in mutagenicity-based assays with similar findings in animal carcinogenicity assays. However, claims in the literature are conflicting. If applied to compounds not specifically selected for their carcinogenic potential, the correlation could be considerably below or be as high as 65% to 85%, if applied to predominantly active compounds, with some 15% to 35% errors as either false positive or false negative findings. This is probably too large a margin of error to make acceptance of mutagenicity-based assays the sole basis for deciding the fate of a substance vis-a-vis the societal environment. The problem of correlation therefore requires considerable further investigation including adequate follow-up studies in intact animals. Many chemical carcinogens have now been found to be mutagens although not all mutagens are carcinogenic.

Despite the existing evidence for a good correlation between carcinogenic and mutagenic activity it is to be noted, that all presently available mutagenicity tests only cover the DNA damage stage of carcinogenesis. They do not deal with the amplification stage, e.g. the differential growth, or the identification of the first transformed cells. Some proposed short-term carcinogenicity tests are not specifically concerned with mutagenic events but are designed to detect other mechanisms leading to the full carcinogenic response (Stitch et al., 1975). A recent large survey emphasized the marked differences in mutagenicity as a function of chemical class (Rinkus & Legator, 1979; 1979a).

The possibility should not be excluded that other disorders, such as autoimmune disease, atherosclerosis, dental caries, the process of aging, etc., may involve the clonal multiplication of variant cells. Some carcinogens, on the other hand, may not operate directly by DNA mechanisms e.g. asbestos and hormones, and therefore give rise to negative results in mutagenicity assays based on detecting DNA damage. Moreover, it is impossible in mutagenicity assays to duplicate the concentration of the ultimate reactive metabolite, organ-specific release, biological half-life, organ specific DNA repair or replication frequency and immunosurveillance.

If in a dividing cell DNA accumulates, there will be no expression of any DNA alteration, until the cell divides into daughter cells with different genetic information, say as a result of translocation. Thus deletion of genetic information in a daughter cell, merely as a result of cell division having occurred, may set up a neoplastic focus. An example of this process is the induction of liver tumours by partial hepatectomy. A carcinogen may therefore be a compound which stimulates cell division, without being responsible for the primary

pathological change, such as inadequate repair of cell damage, which then leads to tumour production.

Although eventually *in vitro* cell transformation in cell culture may substitute for animal carcinogenic bioassay, and certain mutagenicity tests are valuable prescreening procedures for carcinogenicity, the ultimate test for carcinogenicity remains the development of a histologically verified tumour in the whole animal on exposure to the carcinogen.

In relation to carcinogenic hazards, the evaluation of single substances should not be based entirely on short-term mutagenicity and *in vitro* carcinogenicity tests but only on appropriate animal bioassays. At present the Salmonella reversion test (Ames test) has been most extensively validated for its high correlation in many chemical classes with the presence or absence of carcinogenic potential. However, the poor correlation with certain chemical classes suggests that other tests should be used as well. Current information points to reasonably good correlations, despite incomplete validation, between carcinogenic potential and mutations in animal tests determining cytogenetic end points with gene mutations in other microbial systems and in cultured mammalian cells, with *in vitro* cell transformation, with the induction of sex-linked recessive lethal mutations in Drosophila, with the results of tests for unscheduled DNA synthesis in mammalian cells, and possibly also with the results of tests for sister chromatid exchanges. The selection of additional tests will depend on the availability of technical resources and the objectives of the test program. For example, improved predictivity has been demonstrated for various combinations of the Salmonella reversion assay with other systems, particularly with *in vitro* cell transformation assays. A positive outcome, by accepted criteria, in a microbial and/or mammalian cell point mutation assay coupled with a clearly positive *in vitro* cell transformation test must therefore be regarded as strong presumptive evidence that the substance under test has a carcinogenic potential, particularly if evidence of primary DNA damage is also available. Most appropriately a battery of tests is used for establishing presumptive evidence for carcinogenic potential.

There is, however, no definitive evidence that the efficiency of detecting chemical carcinogens would improve by the continued addition of a greater number of assays. It has been shown that a larger number of tests increases the percentage of carcinogens giving a positive result in at least one of the assays used, but simultaneously the percentage of non-carcinogens giving a positive outcome in at least one of the tests also increases (Purchase et al., 1978).

The reported correlation between mutagenic potency in Salmonella reversion assays and carcinogenic potencies (Meselson & Russell, 1977; Ames & Hooper, 1978) promoted the view that short-term tests may eventually be used to predict carcinogenic potency in humans. Evidence on this point is conflicting, the data in all these studies being derived from relatively small number of chemical carcinogens not adequately representing the different chemical classes.

If genetic toxicity testing of a chemical reveals evidence of somatic rather than germinal toxicity it is an indication for treating the substance as a carcinogen for purposes of human risk assessment. A definitive cancer bioassay would be required. It should be borne in mind, however, that the quantitative extrapolation from traditional animal bioassays to cancer induction in a heterogenous human population is still largely unresolved.

One view, based on a mechanism of action for chemical carcinogens involving point mutations in the genome of a normal somatic cell, transforming it into a cancer cell, holds that theoretically one molecule of a mutagen/carcinogen can then cause one such event in a single cell. One cancer cell can, in theory, then develop into a whole tumor. Others hold the view that the assumption of a no-threshold situation for chemical carcinogens disregards the effectiveness of DNA repair, and the immunocompetence of the host which may

enable him to protect himself and reject the incipient abnormal cells. If these latter arguments are correct, then the induction of mutagenesis/carcinogenesis may be regarded as a dose-dependent phenomenon with a threshold value.

Selecting Batteries of Testing Procedures

Biological indicator systems of sufficient sensitivity are needed to detect substances having a mutagenic and/or carcinogenic potential in food at low levels. Assuming availability of these tests systems, the mutagenic fingerprint characterizing the food ingredient under examination can be established to complement the metabolic, chemical (specification) and toxicological fingerprints. In addition, some of the indicator systems would permit tracing the fate of any inherent mutagenic activity, or its appearance *de novo,* of a food ingredient as the latter passes through the phases of intraluminal digestion and metabolism, absorption, distribution, host metabolism, and excretion.

The dynamic state of the mutagenicity testing field implies that new procedures, and modifications of established tests, are constantly being evolved permitting the satisfactory demonstration, mechanistic elucidation and evaluation of action on the exposed genome by a chemical under investigation. To date, experience with any of the available tests systems is limited. Many variables still exist, as in all biological sciences, which defy exact definition and control. As regards the validity and reliability of these test procedures, reference should be made to the introduction. However, many large scale validation exercises are presently being conducted (NCI *In vitro* Carcinogenesis Program, Environmental Mutagen Test Development Program at NIEHS, National Toxicology Program).

It is obvious that no single fixed array of mutagenicity tests will be suitable for all evaluative purposes because the potential value for extrapolation of results to man varies for the different screening procedures. Flexibility in the selection of tests is necessary depending on the particular objective to be studied or evaluated. Therefore if the objective is the ascertainment of mutagenic potential rather than the qualitative assessment of the spectrum of mutagenic activity, or when a rough estimate based on moderate, high and low categorization of the mutagenic potency is needed as a basis for human risk evaluation (EEMS, 1978), or when the battery is deployed to screen large numbers of chemicals which cannot be tested in laboratory mammals.

The composition of the battery and the sequence of testing procedures selected will depend on the problem area being considered, whether it is to be used to predict possible carcinogenic activity or as part of the sequence of testing in product development, in setting priorities for selecting chemicals for long-term bioassays or for the hazard rating of industrial chemicals.

A test system for predicting mutagenicity or carcinogenicity via an endpoint other than carcinogenesis in a mammalian bioassay should, as far as possible, meet the following criteria, if a claim for its inclusion in a test battery is to be upheld.

A. Optimal criteria for selection:
   1. Good predictive capacity established by validation studies. These should demonstrate a strong empirical correlation between induction of the particular test endpoint and of carcinogenesis. A wide range of chemical classes, including both carcinogens and non-carcinogens, should have been surveyed. Conduct of the tests should include the use of coded compounds and predefined test protocols. Results should be interpreted by predefined criteria to classify responses as positive, negative or ambigous.
   2. Reasonable theoretical basis relating the endpoint of the test to mutagenesis or carcinogenesis, supported by experimental evidence.
   3. Good reproducibility between laboratories and in intralaboratory calibrations.

The endpoint of the tests should be of an objective nature and adequate sensitivity.

B. Auxiliary criteria for selection
   1. The test should be rapid compared with the typical mammalian cancer bioassay.
   2. The test should be modest in its demands on resources, including scientific personnel, physical facilities, materials and test mammals, where relevant.
   3. The test should respond to very small quantities of active substance by adequate sensitivity.
   4. The test should be able to predict specific human carcinogenic potential, but this is currently not achievable despite its considerable importance.

C. Practical experience
   1. Many of the test systems are based upon mutagenicity, and are therefore to some extent predictive of the potential induction of heritable genetic disease. This capacity, and the corresponding interpretation of test results, is important.
   2. The early state of development of short-term carcinogenicity testing inevitably implies that virtually none of the available tests meets the entire set of criteria, e.g. the predictive value of tests has most often been validated by uncoded samples.
   3. A number of chemicals have been reported to be inactive in bacterial tests but active in either mutation or transformation tests in cultured mammalian cells and vice versa. These results, together with the important differences in chromosomal structure between prokaryotes and eukaryotes, underline the need to use tests from both phylogenetic categories. Moreover, large multilocus deletions in eukaryotes are not detected in prokaryotic tests, resulting in erroneous qualitative and quantitative judgements.
   4. Even structurally related chemicals within a chemical class possess marked differences in mutagenicity.

Test strategies are functions of the basic purposes of screening and fall into two broad categories:

1) Tier testing proceeds through a series of tests of increasing complexity in phylogenetic sequence. This order is neither absolutely necessary nor always most desirable. For example, mutagenicity screening passes from bacteria, through microbial eukaryotes, insects and/or cultured mammalian cells to *in vivo* mammalian systems. Normally compounds exhibiting activity at a given level may advance to the next level. Errors arise from inappropriate sensitivities or specificities of the selected assays for particular chemical classes.

2) Battery testing is applied to a chemical of interest and uses a predetermined set of tests simultaneously for assessment of presumptive evidence of mutagenic or carcinogenic potential. The efficacy of the battery depends critically upon the selection of individual components, so as to achieve a high predictive capacity among the relevant chemical classes. The battery should also cover as many types of genetic lesions as possible and cover all likely metabolic products. The limited information on validation and standardization suggests the need for deliberate redundancy in test deployment. If the purpose is to improve the overall correlation between activity in the test battery and mammalian, including human, carcinogenic potential, then selection must take into account the performance of structural analogs of the test chemical in the various candidate tests. In the absence of such information, the empirical correlations established in formal validation studies using many classes of chemicals must be relied upon. In general the use of a battery of tests lessens the chance of missing a false negative result or relying on a false positive outcome.

If the requirement is the screening of large numbers of compounds, either for selecting those to be accorded priority for more extensive testing or as part of early product development, then a battery of tests will identify the potential genetically active candidates.

It should be remembered that all of the prac-

tical procedures for detecting mutagenic activity can be viewed as short term procedures when compared to chronic toxicity tests.

Interpretation of Test Results

The major problem area in genetic toxicology which is unresolved is risk estimation from the data generated in tests on laboratory organisms. The present state of the art only allows, at best, categories of concern to be established relating to the potential mutagenic risk for man. The mutagenic potency of a chemical may be defined in this context as the anticipated genetic damage to the human population exposed to a given dose and in these circumstances measures the potential risk to man. This extrapolation to man is based on the assumption of the similarity in DNA and chromosome structure between man and laboratory animals. Mutagenic potency as defined above is then a function of pharmacokinetics (absorption, excretion, transport of chemical and/or its metabolites to and across target cells [particularly the effective concentration in spermatogonia and oocytes], metabolism in target cells, opportunity to react with DNA or other marcomolecules in target cells, and of intrinsic mutagenic potential interaction of active form with DNA, fixation of lesion as stable DNA change through repair or replication, transmission to subsequent cell generations).

Additional factors to be considered in any assessment of the potential risks entailed by the use of a mutagenic substance in comparison with expected benefits should include: the size and type of population exposed, the degree of exposure, the possibility of controlling usage so as to restrict exposure to the minimum, the existence of species differences with regard to metabolism and pharmacokinetics, the lack of possible urinary or biliary excretion effects in *in vitro* systems with consequent possible longer action than under *in vivo* conditions, the alteration of spatial distribution of enzymes as a result of homogenization procedures, differences in co-factor concentrations between *in vitro* and *in vivo* situations, the variability in activation capability and enzyme profile of human liver as determined genetically or as a result of inducer action. Therefore, serious difficulties arise when relying for decision-making entirely on short-term tests, particularly those using *in vitro* approaches only, and in extrapolating experimental results from *in vitro* and *in vivo* systems to man.

The problem is compounded if risk evaluation has to be made of complex mixtures of the kind represented by many foods, because no agreed procedures exist at present for making quantitative assessments of the mutagenic risk arising from the presence of a substance in food. Particular difficulties arise when assessing the genetic safety of natural food components or of products generated in food during processing. It is frequently not possible to isolate these substances without thereby altering their chemical structure, which latter is often unknown. Moreover, neither these food components nor whole food itself can be tested in concentrations offering adequate margins of safety, thus requiring testing procedures with very high resolving power (Ehrenberg, 1977a).

In assessing the consequences of exposure to a mutagen all types of effects on the genome need to be considered and where possible the risk for each event has to be assessed separately. In the absence of specific information on comparative metabolism and pharmacokinetics it must be assumed that the chemical will show the same behavior in man as in the test animal. However, the results of microbial assays used for screening purposes do not allow the evaluation of the effects in man of chronic exposure to the substances tested.

In order to achieve some measure of categorization of concern related to potential mutagenic activity in man it is important that the precision of any tests used be such as to detect doubling of the control level of mutations with a statistical significance at the 5% level. A negative outcome of tests is not acceptable if the false negative errors involved are too large, although these errors may be reduced by examining more end-points. Both

concurrent and historical control data for spontaneous mutation frequencies are needed in the establishment, where possible, of dose-response tabulations. It is to be noted, however, that very few data exist on dose-response relationships for gene mutation induction in intact mammals. Mutation frequency rates are also best quoted numerically and not as a percentage.

Since biochemical data are not very sensitive and selective, the best approach for categorizing the concern related to the human genetic risk from chemicals with mutagenic potential, which cannot be removed from the environment, is to measure their genetic effect after the administration of the chemical by the appropriate route to the intact mammal. For example, estimations of the frequency of gene mutations may require definitive mutation bioassays such as assays determining effect on meiotic cells, assays for dominant skeletal mutations, heritable translocation assays or the mouse specific locus test. The latter test however does not differentiate point mutations from short deletions and thus probably measures primarily short deletions, making the test less sensitive. No method exists for determining the potential mutagenic risk to man from base pair substitutions or frame shifts, the effects being frequently recessive, and then not detectable in the human population with consequent accumulation over many generations. However, it is known that, for example, ring alkylation of DNA purine or pyrimidine (e.g. N-7 guanine) is less likely to cause mutations than alkylation at the functional NH_2 or OH groups (e.g. guanine 0-6), where the rate of repair is much slower.

Chromosomal aberration tests such as *in vivo* cytogenetics, micronucleus or dominant lethal tests are not definitive mutation bioassays and should not be used for estimating the potential for germinal genetic toxicity. If categorization of concern is attempted, the nature of the chromosomal effects and their frequency, visible at meiosis in both oocytes and spermatocytes, in addition to mitotically observable changes must be known. Test systems using intact mammals, including the testing of body fluids for active metabolites, are essential. Examples are determination of X-chromosome loss, nondisjunction or breaks in mice germ cells or heritable translocations in terms of sterility or semisterility of progeny. No useful tests exist at present to find the rate of induction of other chromosomal aberrations and the significance of sister chromatid exchanges for human genetic events is not known at present. Difficulties also arise because many potent mutagens are known to produce negligible chromosomal aberration frequencies in mice spermatogonia. Possibly the post-meiotic stages may be up to 1000 times more sensitive to translocation induction than spermatogonial stem cells. If this is so, then, despite the short duration of the post-meiotic stages, their higher sensitivity to translocation induction may make these a transmissible risk. Similarly, the expected frequencies of translocations in human germ cells cannot be correlated with the frequency of chromosomal aberrations in cultured peripheral lymphocytes or other somatic cells. Only comparison with the incidence of malignancy at a later age could help establish any correlation.

Other difficulties may arise with chemicals active *in vitro* but not in the intact mammal. Furthermore, most active substances induce both chromosomal aberrations and point mutations, while some chromosomal events are not passed on in meiosis.

Point mutational effects are potentially hazardous to man, chromosomal damage in mammalian cells being probably hazardous. Hence categorization of any substance considered to be potentially mutagenic by accepted criteria requires *in vivo* testing. In addition, information has to be provided regarding its toxicological profile, its pharmacokinetic and its environmental behaviour (ICPEMC, 1979). Evidence should be sought whether the substance reaches the germ cells and produces an effect including induction of unscheduled DNA synthesis or binding to germinal DNA. Information is also needed from chemical assays on blood, urine, bile and other body

fluids, as well as on gonadal and other target tissues for the local concentration of the active agent. It is therefore advantageous to carry out the investigations for as many genetic endpoints as possible simultaneously in the same animal. This allows correlation of the various endpoints under the same test conditions (Legator, 1977).

Dose-response relationships established in assays based on cytogenetic endpoints, coupled with various assumptions relating chromosomal changes to point mutations in germinal tissues, may permit the derivation of some estimates of likely resultant alterations in mutation frequencies due to these genetic events. In arriving at such estimates account must be taken, however, of the relationship of the dose causing a mutagenic event in the laboratory model or test animal to the highest likely human exposure.

Recently an approach has been suggested in which chemical data on body and tissue levels have been related to the induction of mutations in microbial indicator systems by blood and urine of animals or individuals exposed to the test substance. Similar correlations have been established with the degree of alkylation of body macromolecules as a measure of germinal DNA alkylation (Legator, 1977).

In risk assessment from animal data two approaches have been described in the literature. The effect on the biological system has been expressed in terms of radiation equivalence. However, the differences in biological activity and dose-response between radiation and chemicals argue against this approach. A second approach has sought to express the response in terms of relative increase in mutation frequencies over control. The above considerations clearly establish that the interpretation of mutagenicity assays is a complex matter and that the decision, whether or not a substance is to be regarded as a mutagenic risk for man, depends on the totality of the information available. In some cases the decision is simple but in the majority of cases, particularly if the results are conflicting, equivocal or difficult to interpret, the decision is dependent almost entirely on the experience and knowledge of those evaluating the data. The most realistic approach may be to acknowledge the limitations of existing data from present short-term tests for purposes of risk estimation. However, once a chemical is judged to have mutagenic potential and has been shown to reach germinal tissues, then the extent of use becomes a social, economic or engineering decision.

Conclusions and Recommendations

DETERMINATION OF MUTAGENIC/CARCINOGENIC POTENTIAL

The assay procedures discussed in this report have been selected to provide evidence on the mutagenic and possible carcinogenic potential of any chemical liable to become an ingredient of food. Some of these tests address the first of these objectives, others the second objective, while yet others address both objectives. In the present situation a deliberate choice of tests has to be made to solve the problem of determining the mutagenic/carcinogenic potential of a substance.

Consequently, for food safety assessment, a core battery of assays is suggested having the following composition:

1. Assay for induction of point mutations by microbial cell systems incorporating *in vitro* activating systems.
2. Assay for induction of point mutations in cultured mammalian cells incorporating a mammalian activating system.
3. Assay for induction of chromosomal changes *in vitro* in cultured mammalian cells.
4. Tests for induction of chromosomal changes *in vivo* by direct cytogenetic analysis of metaphase and/or micronucleus test.
5. Testing of body fluids of treated mammals using microbial indicator systems.
6. Assay for cell transformation using appropriate *in vitro* cultured mammalian or human cell lines.

As an adjunct to this core battery it may be desirable in future to add a test for sex-linked

recessive lethals in Drosophila and an assay for primary non-specific damage to DNA such as induction of unscheduled DNA synthesis and/or DNA repair in mammalian cells, once these tests have become better validated and standardized. These tests should not be included routinely at present in a core battery.

If further exploration is to be undertaken in order to establish categories of concern with respect to the potential mutagenic risk of the chemical for man, the additional investigations should include:

i) A dominant lethal test;

ii) A test for induction of point mutations by a host mediated assay using microbial or mammalian cells as indicator systems;

iii) Heritable translocation test.

In vitro microbial and somatic cell tests for point mutations with and without metabolic activation, indirect *in vivo* tests for gene mutations (host mediated assays, body fluid analysis), and tests for chromosomal aberrations in intact mammals constitute the essential data base for further decisions. The determination of the occurrence of chromosomal changes might require further exploration by a dominant lethal study, scoring for sperm abnormalities or for chromosomal aberrations in mammalian testicular cells, or a heritable translocation test, particularly if positive results were scored in cultured human peripheral lymphocytes. Additional information on DNA damage would be provided from a study of DNA repair and from evidence for unscheduled DNA synthesis. It would also be desirable to include studies in Drosophila.

Attention should be drawn once again to the caveat expressed earlier in the section on predictive capabilities of existing tests. Any review of the state of the art in the field of mutagenicity testing and any assessments of the value of the various procedures mentioned is, of necessity, incomplete and provisional, considering the dynamic state of the evolution of knowledge in the genetic sciences. Failure to mention a testing procedure, or to allocate it a place in the schemes recommended here, does not imply an adverse scientific judgement *per se* but merely expresses an inability to give a precise evaluation of its role in the present situation. On the other hand, any recommendations made in this review should be regarded as guidelines rather than as rules to be followed rigidly in every circumstance. In future, changes may well become necessary as a reflection of the then current state of the art.

CRITERIA FOR JUDGING THE MUTAGENIC POTENTIAL OF A SUBSTANCE

It is an inescapable conclusion that at least some substances will be misjudged by any array of tests now available, however numerous its constituent components. By the same token, no amount of data will ever be sufficient to prove incontrovertibly that a substance has no mutagenic or, for that matter, carcinogenic potential, just as the establishment of absolute safety of a chemical is impossible. It is therefore unnecessary, and in many instances even inappropriate, to demand the performance of the whole proposed battery of mutagenicity assays for every substance to obtain formal reassurance of safety.

Careful consideration of the chemical structure of the toxicological profile as manifested by animal data, and of the metabolic fate of a substance may make testing for mutagenic potential inappropriate. Such judgement may be buttressed by existing epidemiological evidence, knowledge of the likely exposure of the general population, or of critical population groups. Similarly, incontrovertible evidence of a chemical or its metabolites not reaching germinal tissues makes pursuit of further mutagenic testing unnecessary.

If for a particular substance the results of the above 6 tests, listed in the proposed core battery of assays, are negative and the false negative errors sufficiently small as a result of adequate design and appropriate choice of criteria for judging the outcome, the substance may be regarded as not having any mutagenic potential.

If one or more of the *in vitro* bacterial tests are positive by accepted criteria, additional inves-

tigations are required, because the results of these bacterial tests are by themselves insufficient to adjudge the compound to have mutagenic potential in the intact mammal. *In vitro* positive findings may however be disregarded, if there is adequate evidence to indicate *in vivo* metabolic inactivation of the compound.

If there is evidence of *in vivo* unscheduled DNA synthesis or if any assay for mammalian cell transformation in an appropriately validated system produces a positive result, chronic testing of the substance in a cancer bioassay is needed. Further exploration by mutation bioassays is not necessary unless the cancer bioassay gives negative results. If the substance is regarded as a potential carcinogen because tests suggestive of somatic genetic toxicity (cell transformation, *in vivo* unscheduled DNA synthesis) are positive, the decision will depend on the outcome of a definitive cancer bioassay.

However, in the event that the positive mutagenic findings, considered as evidence for potential carcinogenicity, are not confirmed by a positive outcome in carcinogenicity bioassays, the substance must be considered as a mutagen and a determination of its potential risk to man must be undertaken. No procedures, or combination of procedures, at the present time can be considered adequate for quantitative mutagenic risk extrapolation from experimental systems to man. One can, however, establish three categories of concern with respect to potential mutagenic risk to man.

Establishment of Categories of Potential Mutagenic Risk

For any categorization of mutagenic risk, in addition to the biological information, the essential requisites are information on the biochemical and pharmacokinetic behaviour of the compound and evidence of its reaching the germ cells:

CATEGORY A

Chemicals can be placed into this category, based upon the recommended battery, if multiple tests including *in vitro* tests in pro- and eukaryotic systems as well as more than one *in vivo* test are positive with adequate evidence of dose response relationship.

CATEGORY B

Positive outcome in any *in vitro* test for either point mutations or chromosomal abnormalities and any single positive result of *in vivo* tests by established criteria, including a clear dose response relationship. To confirm that the chemical is in Category B the following additional tests should be conducted and be negative: dominant lethal test, germ cell cytogenetics, heritable translocation. If any of these additional tests is positive the chemical falls into Category A.

CATEGORY C

i) Positive *in vitro* findings in prokaryotic and eukaryotic systems only.

ii) Marginal but statistically significant activity at high concentrations as the outcome in only one of the *in vivo* tests which include the additional tests mentioned under Category B, and where a dose/response relationship cannot be established.

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Quantitative Risk Assessment

This review considers the issues addressed by the Scientific Committee of the Food Safety Council related to risk assessment as a part of their four year task to study the body of knowledge regarding food safety testing, and to recommend appropriate criteria to measure the risk of food ingredients. The Committee concludes its evaluation of existing models with recommendations. However, as noted, the choice of mathematical procedures for low-dose extrapolations has no firm biological basis and to some extent is arbitrary. The summary of the selection of the most appropriate model is concluded at the end of the review.*

Introduction

1. This review considers the problems involved in quantitatively assessing the implications for human risk of whatever evidence for acute and/or chronic toxicity, mutagenicity and/or carcinogenicity has emerged from the testing program. Such evidence or the absence of evidence will have been found under special laboratory conditions, such as tests on limited numbers of animals at exposure levels well above the contemplated use levels, so that the applicability of the results to humans is by no means automatic or immediate. As Paracelsus observed, "All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing a poison" (Paracelsus, 1493-1541). Small amounts of ingredients that are harmful in high doses are now known to be present in many natural foodstuffs such as cabbage which contain goitrogens, roast, grilled or charcoal-broiled meats, which contain the carcinogen 3-4 benzopyrene, and potatoes, that contain the mitotic poison solanine (Golberg, 1967). The rough, intuitive experience of mankind has been that even repeated exposure to low doses of substances which are toxic at high doses need not be harmful and may even, as in the case of arsenic, lead to the development of resistance to the high dose effects. This review outlines the efforts which have been made to refine this intuitive experience by use of appropriate mathematical models, with a view to recommending a risk evaluation procedure that is appropriate in the light of current knowledge.

Human risk assessment is a very inexact exercise, based largely upon theoretical assumptions concerning interspecies extrapolations. The uncertainties involved should be fully recognized by the scientific community and society. The methods used by all the mathematical models attempt to predict how many test animals will respond at low exposure levels, based upon observed responses at high dose levels. The models tell us little about predicted human responses at any exposure level. This requires judgmental decisions based upon broad biological assumptions, or comparative metabolic data, if it is available. Quantitative risk assessment can, at best, provide a range for the incidence of potential toxic effects in humans.

Furthermore, before methods of quantitative risk assessments are even considered, the qualitative nature of the biological data used to predict human risk must be taken into account. The extent and validity of the data may determine which of the mathematical models is more appropriate, or if the use of any model is justified. The complex tests required before a human risk assessment can be considered, allow for many possible errors of procedure or judgment, any of which could have major ef-

*This paper is a revised version of Chapter 11 of the Committee's report "Proposed System for Food Safety Assessment", originally published in December 1978 as Supplement 2 of Volume 16 of *Food and Cosmetics Toxicology*. The report is available from the Food Safety Council, 1725 K St., N.W., Suite 306, Washington, D.C. 20006, U.S.A.

fect upon quantitative predictions. It should be stated at the outset, that inadequate or questionable animal test data should preclude the use of any mathematical models, since the results are likely to be totally misleading. Such data should be considered as preliminary or perhaps serve as the basis for purely a judgmental assessment of risk if additional biological factors such as those listed in Table 1 are available and add to or corroborate the animal findings.

Mathematical Models

2. All models start with an experimental determination of response at several dose levels of the agent of interest in one or more species of laboratory animals regarded as relevant to human risk evaluation. We shall use "response" as a general term covering acute and/or chronic toxicity, mutagenicity and/or carcinogenicity. Such a set of experimental results is conceptualized mathematically as a sampling from an underlying dose-response curve, which relates the probability of some well-defined response to the dose administered.* From such data and the inference to the conceptualized dose-response curve it is required to determine an acceptable exposure level. Probably the earliest and perhaps still the most widely used way of doing this involves the determination of a "no observed effect level," i.e. the dose at which none of the experimental animals respond. But this dose level is markedly dependent on the number of animals tested, 0 out of 10 and 0 out of 100 clearly having different interpretations. It also assumes the existence of a threshold below which no response will take place, no matter how many animals are tested. We shall return to the threshold concept later in the chapter, but it is clear that even if one accepts it, the equating of the "no observed effect level" and the "no effect level" is statistically naive and that more careful treatment is required. This treatment must involve at a minimum a specification of a theoretical dose-response curve and a procedure for estimating its parameters from responses at all dose levels.

Most models express the probability of a response P, as a function, f, of dosage, D, so that $P = f(D)$ and differ only with respect to choice of function, f. Non-threshold models assume that if proportion p of control animals respond, that $f(D) = p$ only for D equal to zero, and that for any nonzero D, $f(D) > p$. Threshold models assume the existence of a D_0 such that for all $D \leq D_0$, $f(D) = p$. If safety is defined as zero elevation over control response, then a non-threshold model would require that any non-zero dosage be deemed unsafe, as in the Delaney clause for carcinogenic responses. With this definition of safety, foods containing residues below the limit of analytic detectability and hence not distinguishable from foods containing no residues, must be deemed unsafe.

The concept of virtual safety, introduced by

TABLE 1
BIOLOGICAL FACTORS TO BE CONSIDERED IN HUMAN RISK ASSESSMENT

Evaluation of Chronic Cancer Bioassay Data
- Number of Species and Strains Affected.
- Number of Tissue Sites at Which Tumors Occur.
- Latency Period.
- Dose Level and Duration of Exposure Required to Induce Tumors (potency).
- Proportion of Malignant vs. Benign vs. Pre-Neoplastic Changes.

Evaluation of Characteristics of the Compound
- Chemical Similarity to Other Known Carcinogens.
- Metabolic and Pharmacokinetic Data.
- Binding to DNA, RNA and Protein.
- Physiological, Pharmacological and Biochemical Properties.
- Genotoxicity and Activity in Short-Term Tests for Carcinogenicity.

Population At Risk
- Age
- Sex
- Physiologic State
- Conditions of Exposure

*Some models consider time to response as well (See Section 2e), but since their implementation requires systematic serial sacrifice, and this is generally not done experimentally, we shall not consider them in detail.

Mantel and Bryan (1961) has provided a way out of this dilemma and has been adopted by the Food and Drug Administration (Federal Register, 1977). A dosage D_0 is said to be virtually safe if $f(D_0) \leq p + (1-p)P_0$, where P_0 is some near-zero lifetime risk, such as 10^{-8}, the value proposed by Mantel and Bryan or 10^{-6}, the value adopted by the FDA. The virtually safe dose (VSD) is then calculated as $f^{-1}[p + (1-p)P_0]$. The calculation thus requires choosing a model, f, determining the value of its disposable constants from observations in the observable range and extrapolating down to the unobservable elevation in response, P_0, to determine the VSD. Specification of a value for P_0, such as 10^{-8}, involves a judgment that is best left to regulatory authorities, so that in what follows we shall be concerned with estimation of P for an array of doses in the low dose range. From this array, a VSD can be computed but we shall not consider the additional judgmental issues required to compute it. In the United States, an elevation of lifetime risk of 10^{-6} for any one substance would amount to 3 additional cases per year.

It is hardly necessary to emphasize that mathematics cannot rise above its source. The application of such procedures to the results of poorly conducted experiments, with inadequate pathologic appraisals, unsatisfactory animal care and unreliable records, will not result in reliable risk assessments. It is necessary that the pathologist and toxicologist provide the biological judgment as to which of the experimental data are appropriate for use in the mathematical model. Data may not be suitable if the quality is questionable, does not represent an appropriate biological sample, or if the nature of the data is not considered relevant to human risk assessment. It is assumed that experimental results have been screened and that only experiments in which sound scientific procedures have been followed will be subject to analysis.

In what follows we do not distinguish sharply between carcinogenic and non-carcinogenic responses, as do some authorities. A major reason often given for making such a distinction has been the existence of individual thresholds for the latter, but not the former response, leading to a functional difference for the two types of responses. But the individual thresholds cannot all be identical, and once the existence of a distribution of individual thresholds for non-carcinogenic responses is admitted there appears to be no practicable way of determining the minimum of this distribution. In particular, the application to observed no effect levels of arbitrary safety factors which disregard numbers of animals and the slope of the dose-response curve does not seem appropriate. Many of the determinants of the functional form of the dose-response curve will be the same for both types of responses. In particular, metabolic activation and deactivation reactions will characterize both types of response and the functional form of the dose-response curve must reflect them (Section 2f). This is not to deny that some non-carcinogenic responses lack the chronic, progressive and irreversible features of the carcinogenic response (some share them), but only that in the light of present knowledge the differences seem more likely to reflect detailed differences in the kinetics of the reactions involved and that if there are differences of kind not enough is known about them to permit an inference to the different kinds of dose-response urves they imply. Mathematical models of carcinogenesis as a multi-stage stochastic process have provided a possible basis for this distinction, but they lack experimental confirmation.

a. *The Probit Model.* This model assumes that the log tolerances have a normal distribution with mean μ and standard deviation σ. The proportion of individuals responding to dose D, say P(D), is then simply

$$(1)\quad P(D) = \Phi[(\log D - \mu)/\sigma] = \Phi(\alpha + \beta \log D),$$

where $\Phi(x)$ is the standard normal integral from $-\infty$ to x, $\alpha = -\mu/\sigma$ and $\beta = 1/\sigma$. This dose-response curve has P(D) near zero if D is near zero and P(D) increasing to unity as dose increases. A plot of a typical probit dose-response is given by an S-shaped curve. The

quantity β in (1) is referred to as the slope of the probit line, where

(2) $Y=\Phi^{-1}[P(D)] = \alpha+\beta \log D$

and Y + 5 is the probit of P.

This model was originally introduced in the context of drug standardization, in which responses in the 5 to 95% range are of most interest so that it was mathematically convenient to assume no threshold for the individual tolerances (Finney, 1952). One does not expect, however, to see 6-inch-tall men or human livers in the picogram range, despite the lognormality of human height or liver weight in the central part of the curve, so that there is no compelling reason to assume that all lognormal distributions start at zero. In other applications of lognormal distributions in which low probabilities are of interest, nonzero thresholds are assumed and estimated from the data (Cohen, 1951; Hill, 1963). The issue of a threshold for individual tolerances does not seem capable of a purely observational resolution for dose-response curves, however, and the shape assumed at low doses must depend on theoretical considerations as well. Despite its nonthreshold assumption it is a characteristic of the probit curve that as dose decreases, zero response is approached very rapidly, more rapidly than any power of dose. Other curves to be considered approach zero response more slowly.

An alternative derivation of the probit model which relates it to time to response, has been given by Chand and Hoel (1974) using the Druckrey observation that median time to tumor, T, is related to dose, D, by the equation $DT^n = C$, where n and C are constants unrelated to D (Druckrey, 1967). Combining this relation with an assumed lognormal distribution of response time then gives the P(D) of equation (1) as probability of response to any given time, T_0, where the α and β of equation (1) are simple functions of n, C, T_0 and the standard deviation of the distribution of response times.

b. *The Logit Model.* This model, like the probit model, leads to an S-shaped dose-response curve, symmetric about the 50% response point. Its equation is:

(3) $P(D) = 1/[1 + \exp\{-(\alpha+\beta \log D)\}]$.

It approaches zero response as D decreases more slowly than the probit curve, since

(4) $\lim_{D\to O}[P(D)/D^{\beta}] = \text{constant}.$

The practical implication of this characteristic is that the logit model leads to lower VSD than the probit model, 1/25th as much in a calculation reported by Cornfield, Carlborg and Van Ryzin (1978), even when both models are equally descriptive of the data in the observable range.

c. *The One-Hit Model.* The one-hit model has a dose-response curve,

(5) $P(D) = 1 - \exp(-\lambda D)$,

where λD is the expected number of hits at dose level D. Hoel, Taylor, Kirchstein, Saffiotti and Schneiderman (1975) have argued that this model is consistent with reasonable biological assumptions. But the concept of a hit is a metaphor for a variety of possible elementary biochemical events and the model must be considered phenomenologic rather than molecular. The model is essentially equivalent to assuming that the dose-response curve is linear in the low-dose region. Thus, the slope of the one-hit curve at dose D is $\lambda[1-P(D)]$, and for dose levels at which $P(D) \leq .05$ varies by less than 5%, i.e. is essentially constant and equal to λ. The linear model is one of two models, the other being the probit model, specified by the Environmental Protection Agency (1976) in its interim guidelines for assessment of the health risk of suspected carcinogens. The assumption of low-dose linearity will generally lead to very low VSD, so low as to lead the Food and Drug Administration Advisory Committee (1971) to remark that assuming linearity ". . . would lead to few conflicts with the results of applying the Delaney clause." The one-hit model, having only one disposable parameter, λ, will often fail to provide a satisfactory fit to dose-response data in the observ-

able range. Other models described below, by introducing additional parameters, often lead to reasonable fits in the observable range.

d. *The Gamma Multi-Hit Model.* This model is a generalization of the one-hit model, as is seen from the following argument. The one-hit model is obtained by assuming that with an expectation of λD hits at dosage D, the probability of exactly x hits is given by the general term of the Poisson distribution, $P(X=x)=e^{-\lambda D}(\lambda D)^x/x!$. If only one hit is required to produce a response, then it can be shown that

$$P(D)=P(X\geq 1)=1-\exp(-\lambda D), \tag{6}$$

the one-hit model of equation (5). By exactly the same reasoning (Cornfield et al, 1978), if at least k hits are required for a response, then

$$P(D)=P(X\geq k)=\int_0^{\lambda D}\frac{u^{k-1}e^{-u}du}{(k-1)!} \tag{7}$$

which reduces to (6) when k = 1. Because (7) contains an additional parameter, k, it will ordinarily provide a better description of dose-response data than the one-parameter curve (5). Equation (7) can be further generalized by allowing k to be any positive number, not necessarily an integer. In this case (7) can be described as that dose-response curve which assumes a gamma distribution of tolerances with shape parameter k. We note

$$\lim_{D\to O}[P(D)/D^k] = \text{constant}. \tag{8}$$

Thus, in the low dose region, the equation (7) is linear for k = 1, concave for k<1 and convex for k>1. At higher doses the gamma and the lognormal distributions are hard to distinguish so that the model provides a blend of the probit model at high dose levels and the logit at low ones.

e. *The Armitage-Doll Multi-Stage Model.* The characteristic (8), in which the low dose probability is proportional to the k^{th} power of dose, where k is the number of stages, was considered by Armitage and Doll (1961) to be quite inconsistent with observation. They derived a multi-stage model, which by assuming that the effect of the agent at some stages was additive to an effect induced by external stimuli at those stages, led to a lower power than k for D. Crump, Hoel, Langley and Peto (1976) have recently discussed this model and by assuming additivity at all stages have obtained as an expression for the required probability

$$P(D) = 1 - \exp\left\{-\sum_{i=0}^{\infty}\alpha_i D^i\right\} \qquad \alpha_i\geq 0. \tag{9}$$

Hartley and Sielken (1977) consider this model and time to response as well, obtaining a more general result. For $\alpha_1>0$ these models also imply low dose linearity since

$$\lim_{D\to O} P'(D)=\alpha_1\exp(-\alpha_0) \tag{10}$$

Armitage and Doll cite the data relating lung cancer mortality to previous smoking habits as leading to a linear dose-response curve, but as Cornfield, et al (1978) point out, errors in reporting amount smoked would lead to such a curve even if the true curve were convex. This supports the view that the apparent low-dose linearity in many epidemiologic studies is an artifact of errors in the reporting of dose. Crump, et al (1976) stress the crucial nature of the additivity assumption, pointing out that it can make orders of magnitude differences in the estimated risk associated with the low dose exposure. We examine the additivity assumption later in this review.

f. *The Weibull Model.* Another generalization of the one-hit model is the Weibull model:

$$P(D) = 1 - \exp(-\beta D^m), \tag{11}$$

where m and β are parameters. Note that

$$\lim_{D\to O}[P(D)/D^m] = \text{constant}. \tag{12}$$

Thus, in the low-dose region, equation (12) is linear for m=1, concave for m<1, and convex for m>1. With a typical set of data, the Weibull model tends to give an estimated risk at a low dose which lies between the estimates for the gamma multi-hit and the Armitage-Doll models.

g. *A Simplified Statistico-Pharmacokinetic Model.* This model (Cornfield, 1977) arises from consideration of an agent subjected to simultaneous activation and deactivation reactions,

both reversible, with the probability of a response being proportional (linearly related to) the amount of activated complex. Denoting total amount of substrate in the system by S and of deactivating agent by T and the ratios of the rate constants governing the back and forward reactions by K for the activation step and K* for the deactivation step, the model is, for

$$D>T \quad P(D) = \frac{D - S[P(D)] - y}{D - S[P(D)] - y + K}$$

$$(13) \quad \text{where } y = K[P(D)]T/\{K[P(D)] + K^*[1 - P(D)]\}$$

$$D<T \quad P(D) \cong \frac{D}{S + K\left(1 + \frac{T}{K^*}\right)}$$

These equations follow from standard steady state mass action equations. Thus, at the low dose levels, $D<T$, the dose-response curve is nearly linear, but for deactivating reactions in which the rate of the back reaction is small compared to that of the forward reaction, K* will be quite small and the slope will be near zero. In fact, in the limiting case in which $K^* = 0$ the dose-response curve has a threshold at $D = T$, but since the model is steady state and does not depend on the time course of the reaction, it cannot be considered to have established the existence of a threshold. For $K^*>0$, the dose-response curve is shaped like a hockey stick with the striking part nearly flat and rising sharply once the administered dose exceeds the dose, T, which saturates the system. Because of the great sensitivity of the slope at low doses to the value of K^*/K, and insensitivity at high doses, responses at dose levels above $D = T$ probably cannot be used to predict those below T. This can be considered a limitation of the model, but it can equally well be considered a limitation of high dose experimentation in the absence of detailed pharmacokinetic knowledge of metabolic pathways. The model can be generalized to cover a chain of simultaneous activating and deactivating reactions intervening between the introduction of D and the formation of activated complex, but this does not appear to change its qualitative characteristics. The kinetic constants, S, T, K and K* are presumably subject to animal-to-animal variation. This variation is not formally incorporated in the model, so that the possibility of negative estimates of one or more of these constants cannot be excluded.

h. The Joint Effect of Two or More Agents. All the preceding models but that of Armitage-Doll describe the effect of one agent by itself. But one must inquire whether the increased response attributable to one agent administered alone is affected by the presence of other agents. This is not a question about the biological mechanisms of co-toxicity or co-carcinogenesis but a purely phenomenological question, the answer to which must be couched in mathematical, not biochemical terms. Even so, the issues are more subtle than they at first appear. The question is not, for example, whether two or more different agents cause a higher response than any one agent by itself (unless they are antagonistic they necessarily will), but whether the increased response attributable to one agent is altered by the presence or absence of others. Thus, it could happen that agent A does not affect the increased risk attributable to agent B administered alone, that agent B similarly does not affect the increased risk attributable to agent A administered alone, but that exposure to both is riskier than exposure to either one alone. (Let A be driving 1000 miles and B be swimming in a riptide.) Such joint effects are referred to as independent and the increased response* found in a one-agent experiment will apply in the presence of other agents as well. The well-known Abbott's correction for background response is equivalent to the assumption of independence of background and the agent under the test (Abbott, 1925).

Other models than independence can be formulated. The concern that small doses which are individually virtually safe will summate to produce a total dosage which is not

*Strictly speaking, it is the increased response divided by the probability of not responding to the other agent (See, reference cited in Cornfield et al (1978), equation (14).)

virtually safe is formally expressed by the additivity model, which assumes that the probability of a response to many agents depends only on the sum of the amounts of each present (when the amounts are expressed in common potency units, cited as equation 16 by Cornfield, 1978). This model is consistent with the Armitage-Doll model and leads, as previously noted, to low dose linearity. The theoretical argument for this assumption in carcinogenesis, that all carcinogens operate by a common mechanism and that any one increases some part of an ongoing process (Crump et al, 1976), is far from compelling. The inactivation paths of different agents will generally be different, so that even if one of the activated complexes simply increases what the others are doing, this will not happen until the various inactivation reactions are saturated. In addition, the metabolic activation of one carcinogen may be inhibited by the presence of another, the classic example being the abolition of the hepatocarcinogenicity of 3'-methyl-4-dimethylaminoazobenzene by coincidental administration of another carcinogen 20-methylcholanthrene (Richardson, Stier and Borsos-Nachtnebel, 1952; Clayson, 1977). Furthermore, even if all the activated complexes work by some common mechanism such as mutation on the DNA, they need not affect the same mutation sites with the same probability, as is made clear by the work of Benzer (1961). As is always the case in science, and particularly in biology, actual experimental results are needed to test the different theoretical models for joint effects. Results that are available (Health and Welfare Canada, 1973; Schmähl, 1976; Arcos, 1972) display considerable diversity and suggest that although the additive model may sometimes hold, it is far from being a common case. A factorial study of 24 pairs of known or suspected carcinogens, each at sixteen combinations of dose levels, financed by the National Cancer Institute and conducted by the Stanford Research Institute should cast considerable additional light on these questions. Preliminary results in individual and joint sub-acute MTD's indicate the usual diversity of joint effects for the individual pairs, with antagonism, independence, partial** or complete additivity and other more complicated joint effects all occurring (NCI Report). Results sometimes cited on the interactive effects in man of cigarette smoking, asbestos exposure, inhalation of radon and alcohol consumption do not bear on the additivity which underlies low dose linearity because that kind of additivity cannot be established by observation of the effects of only one dose-level of an agent, as is the case for asbestos, radon and alcohol (Cornfield, 1975). From the regulator's point of view it would appear as if additive joint effects are not so common as to be automatically assumed, but that when their existence, and hence low dose linearity, are suspected, this would influence the choice of mathematical models.

i. Measured Responses. All the preceding models assume a count of the number of positive and negative responses at each dose level. When experimental responses take the form of measured responses, such as time to tumor, serum or liver enzyme levels, or degree of tissue degeneration, dichotomization of the data is necessary. This requires scientific judgment regarding limits of normal values or significant tissue alterations. As far as possible these judgments should be supported by statistical criteria.

Biological Considerations in Risk Assessment

3. While a variety of mathematical models have been discussed the recommendation in favor of any one of these models for all applications cannot be made at this time. Because the mechanisms of carcinogenesis are not understood, even those mathematical models drawing on biological theory cannot claim to be universally correct. Similarly, statistical

**For complete additivity it is necessary that a mixture of one-half the first LD_{50} and one-half the second also lead to a 50% response. An instance of partial additivity would occur when the mixture leading to a 50% response contained some fraction, c_1 of the first LD_{50} and c_2 of the second where both c_1 and c_2 were greater than ½ but less than 1. Partial additivity could be of concern, but unlike complete additivity, it does not automatically lead to low dose linearity.

considerations alone cannot lead to the adoption of one particular model for purposes of risk assessment. Even an optimally designed experiment involving a moderately large number of experimental animals will have only limited power to discriminate between two plausible models (Crump, 1978).

Although many of the proposed models will provide adequate fits to data in the observable range, those same models can yield widely divergent results on extrapolation to low dose levels (Chand and Hoel, 1974; FDA, 1971). A similar conclusion was reached recently by the Commissioner of the Food and Drug Administration in promulgating Criteria and Procedure for Evaluating Assays for Carcinogenic Residues (Fed. Reg., 1977) said:

"The Commissioner has extensively reviewed the known procedures that may be used to derive an operational definition of the non-residue standard of the act from animal carcinogenesis data. This review has persuaded him the same scientific and technological limitations are common to all. Specifically, because the mechanism of chemical carcinogenesis is not understood, none of these procedures has a fully adequate biological rationale. All require extrapolation of risk-level relations from responses in the observable range to that area of the dose-response curve where the responses are not observable. Matters are further complicated by the fact that the risk-level relations adopted by the various procedures are practically indistinguishable in the observable range of risk (5 percent to 95 percent incidence) but diverge substantially in their projection of risks in the non-observable range".

Because of this ambiguity, some experimentalists have rejected the use of mathematical procedures for determining acceptable exposure levels as pseudo-scientific. Regulatory decisions, however, must be made even in the absence of complete knowledge. Decisions based on informed scientific *judgement*, moreover, may be more easily criticized than those based on the systematic application of an *objective* set of decision making criteria which provide insofar as is possible for the biological and statistical uncertainties involved. Thus, the issue does not appear to be mathematical models versus no mathematical models, but the development of orderly and systematic procedures for low-dose risk assessment which utilize all available biological and statistical information.

In the final analysis, the criteria used to select the most appropriate model and risk level must include an assessment of the biological characteristics of the test substance, the nature and severity of the toxic effect, as well as an evaluation of the statistical goodness-of-fit of various mathematical models. In the case of carcinogens, for instance, the biological factors listed in Table 1 should be taken into consideration.

Certain of the more potent animal and human carcinogens share common characteristics, in terms of their biological and chemical activity. For example, it is now well established that carcinogenic substances such as aflatoxin and vinyl chloride are metabolized to proximate electrophilic carcinogens (Miller and Miller, 1976) which alkylate NDA bases. Substances in this class usually are carcinogenic in more than one species, and produce tumors at multiple sites (at least in high doses). In most instances the latency period is relatively short and inversely related to dose level. They usually induce a high proportion of malignant tumors as well as a spectrum of pre-neoplastic and benign changes indicative of carcinogenicity.

Other properties shared by these substances include their activity in short-term tests for carcinogenicity and genotoxicity as discussed in Chapter 6. In this connection, many of the well known electrophilic carcinogens produce positive results in the Ames Test (Workshop, 1979; McCann and Ames, 1976) and are active in transforming cells *in vitro* (Purchase, et al., 1976). For substances possessing these biological characteristics, a conservative approach to risk assessment is warranted.

On the other hand there are substances which induce tumors in animals, for which a less conservative approach to risk assessment is indicated. These substances are not known to be metabolized to electrophilic agents and

neither the parent compound nor its metabolites (if the substance is metabolized) bind to critical cellular components. Often they produce tumors in only one species, only at high dose levels or only under unique test conditions. In fact, the exposure levels which are carcinogenic are also sufficiently high to induce extensive and chronic tissue necrosis and regeneration. For the most part these substances are inactive in the short-term tests for carcinogenicity and show little, if any, evidence of genotoxicity.

Based on certain theoretical assumptions of the mechanisms of carcinogenesis it has been proposed by some authors, (Hoel et al. 1975) that low-dose linearity must be assumed in all cases in the selection of mathematical models for extrapolation of animal tumor data. It is argued that such an approach would minimize potential human risk from exposure to chemicals found carcinogenic in animal bioassays. Even if these assumptions are true, however, low-dose linearity would most likely be limited to classical electrophilic carcinogens, i.e., those acting through a mutation-like event. Such responses would be difficult to imagine with substances which are carcinogenic as the result of prolonged tissue damage. Application of this philosophy in all cases is considered to be unwarranted, and overly conservative, since in some instances low-dose linearity can be excluded on biological or metabolic grounds. Further, such an extreme approach will often unnecessarily preclude the use of substances which are of significant societal or industrial value.

For non-neoplastic effects, similar considerations apply in the choice of mathematical model or risk level. Irreversible brain damage or progressive anemia, for example, would warrant a more conservative approach than would transient and reversible abnormalities such as enteritis.

Methods Available for Low-Dose Risk Assessment

4. There are a number of methods for low-dose risk assessment, all based on the mathematical models discussed in Section 2. The two most common are the Mantel-Bryan (1961) and a procedure based on low-dose linearity (or the one-hit model), but others have been developed.

a. *The Mantel-Bryan Procedure.* As originally proposed, this procedure used the probit model, but with a preassigned slope of unity, the rationale being that all observed probit slopes at the time of the proposal exceeded that value, the procedure therefore being considered conservative. An additional conservative feature involved use of the upper 99% confidence limit to the proportion responding at a dose level, rather than the observed proportion. The procedure then extrapolates downward to a response level of 10^{-8}, using each separate dose level in the experiment, or combinations, taking as the VSD the highest of the values obtained. A conservative method of taking account of the response of the control group is also given. An improved version of the procedure, which includes handling several sets of independent data, and better methods of handling background response rates and responses at multiple doses has since been published (Mantel, Bohidar, Brown, Ciminera and Tukey, 1971)

b. *A Method Based on the One-Hit Model.* Using the one-hit model, one can obtain the maximum likelihood estimate of the parameter λ based on response at various dose levels and from this one can extrapolate backward to obtain the dose at any preassigned risk level. A conservative assessment is given by taking a confidence limit, say the 99% confidence limit for this VSD. Background response can be taken care of by using Abbott's equation (1925). (For the one-hit model independent or additive background effects lead to the same model).

c. *Methods Based on the Armitage-Doll Model.* A recent paper by Crump et al. (1977) gives a procedure for low-dose extrapolation in the presence of background which although based on the generalized model (9), always reduces, when upper confidence limits are used, to extrapolation using low-dose linearity. This is because the use of upper confidence limits on α_1

on the model (9), is equivalent to admitting the possibility of a positive value of α_1, which at low doses dominates the expression (9). This point is discussed in the last section of the paper by Crump et al. (1977) (See also Cornfield, et al. 1978). Once upper confidence limits on the VSD or risk at a given dose are used, there may be little practical difference, therefore, between use of the one-hit model and the generalization given by equation (9).

Hartley and Sielken (1977) have developed a procedure based on maximum likelihood for the Armitage-Doll model. Their program is very general and allows for the inclusion of the effect of the time to a tumor. VSD's and their confidence intervals may also be calculated with this program.

d. *A Method Based on the k-Hit Model.* Procedures for estimating the parameters of the k-hit model by nonlinear maximum likelihood estimation have been developed by Rai and Van Ryzin (1979a, 1980) and a computer program is available in Rai and Van Ryzin (1979b). This method has the advantage of permitting the data to determine the number of hits needed to describe the results without introducing more than two parameters. When only one dose level gives responses greater than 0 and less than 100%, unique values of the two parameters can no longer be estimated. The background effect in this model is taken care of by using Abbott's correction.

e. *A Method Based on the Weibull Model.* The conventional statistical procedure of weighted least-squares provides one method of fitting the Weibull model to a set of data. With a background response measured by the parameter p, the model using Abbott's correction is

$$P = p + (1 - p)(1 - \exp(-\beta D^m)) = 1 - \exp(-(\alpha + \beta D^m)),$$

where $\alpha = -1n(1 - p)$. With the transformation $Y = -1n(1 - P)$, the model becomes

$$Y = \alpha + \beta D^m.$$

With a nonlinear weighted least-squares regression program one can estimate the three parameters (m, α, β) directly. With only a linear weighted least-squares regression program, one can use trial and error on m to find the values of the three parameters which produce a minimum error sum of squares. A program for one electronic calculator (the TI-59) which conveniently handles up to nine data points is available. This weighted least squares program is available from the Food Safety Council.

A nonlinear maximum likelihood method to obtain estimates of the parameters in the Weibull model can also be used. A program for this purpose is available from Dr. Dan Krewski, Head, Chemical Statistics Section, Health Protection Branch, Health and Welfare, Canada. The calculations for the Weibull model in the next section were provided by Dr. Krewski.

f. *A Method Based Upon the Pharmacokinetic Model.* Taking advantage of the similarity of the probit and pharmacokinetic models in the 5% to 95% range, Cornfield (1977) developed an approximate method of estimating its parameters, particularly the value of T, the saturation dose. Risks at dosages below T are crucially dependent on K*, the relative speed of the back deactivation reaction and this cannot be well estimated from responses at dosages above T, so that low-dose assessment using this model may be more dependent on further pharmacokinetic experimentation than on further statistical developments.

Performances of the Gamma, Armitage-Doll, Weibull and One-Hit Models

5. Each of these four models has been fitted to the dose-response data in Table 2 from 14 different experiments with 13 different substances. Nine of the 14 responses are tumors of some kind, and five of the 14 are other than a tumor. The results are given in Tables 3 and 4. Table 3 presents estimates of background response, information about the parameter estimates for each model, a goodness-of-fit p value for each of the models, and p values for the improvement of the Weibull and gamma mul-

TABLE 2
EXPERIMENTAL RESULTS FOR FOURTEEN SUBSTANCES

Substance	Species	Type of Response	Dose Units	Response Data / Dose No. of responses/No. of animals										
1. NTA	Rat	Kidney Tumor	% in diet	0	.02	.20	.75	1.50	2.00					
				0/127	0/48	0/48	1/91	2/91	12/48					
2. Aflatoxin B_1	Rat	Liver Tumor	ppb.	0	1	5	15	50	100					
				0/18	2/22	1/22	4/21	20/25	28/28					
3. Ethylenethiourea	Rat	Fetal Anomalies	mg./kg.	0	5	10	20	40	80					
				0/167	0/132	1/138	14/81	142/178	24/24					
4. 2,3,7,8-tetrachlorodibenzo-p-dioxin	Rat	Intestinal Anomaly (fetuses)	mg./kg.	0	.125	.25	.50	1.00						
				0/24	0/38	1/33	3/31	3/10						
5. Dimethyl-nitrosamine [1]	Rat	Liver Tumors	ppm.	0	2	5	10	20						
				0/29	0/18	4/62	2/5	15/23						
6. Vinyl Chloride[2]	Rat	Liver Angiosarcoma	ppm.	0	50	250	500	2500	6000					
				0/58	1/59	4/59	7/59	13/59	13/60					
7. Hexachlorobenzene	Rat	14th rib Anomoly (fetuses)	mg./kg.	0	10	20	40	60						
				0/80	4/79	8/91	15/87	25/96						
8. Botulinum toxin—Type A[3]	Mouse	Death due to botulism	ng.	.010	.015	.020	.024	.027	.030	.034	.037	.040	.045	.050
				0/30	0/30	0/30	0/30	0/30	4/30	11/30	10/30	16/30	26/30	26/30
9. Bischloromethyl Ether	Rat	Respiratory Tumor	No. of 6 hr. exposures by inhalation of 100 ppb	10	20	40	60	80	100					
				1/41	3/46	4/18	4/18	15/34	12/20					
10. Sodium Saccharin	Rat	Bladder Tumors	% in diet	.01	.10	1.0	5.0	7.5						
				0/25	0/27	0/27	1/25	7/29						
11. Ethylenethiourea	Rat	Throid Carcinoma	ppm.	0	5	25	125	250	500					
				2/72	2/75	1/73	2/73	16/69	62/70					
12. Dieldrin[4]	Mouse	Liver Tumor	ppm.	0	1.25	2.50	5.00							
				17/156	11/60	25/58	44/60							
13. DDT	Mouse	Liver Hepatoma	ppm.	0	2	10	50	250						
				4/111	4/105	11/124	13/104	60/90						
14. Rapeseed (span) oil	Rat	Cardiac Lesion	% in diet	0	5	10	15	20						
				1/10	1/10	4/10	4/10	5/10						

(1) Results at 50 ppm. omitted.
(2) Results at 10,000 ppm. omitted.
(3) Results at .001, .005, .055, .060, .065 ng. omitted.
(4) Results at 10, 20 ppm. omitted.

TABLE 3
RESULTS OF FITTING FOUR MODELS TO FOURTEEN SUBSTANCES

	ONE-HIT MODEL		ARMITAGE-DOLL MODEL			WEIBULL MODEL				GAMMA MULTI-HIT MODEL			
Substance	Estimate of Background	Goodness-of-Fit P Value	Estimate of Background	$\{\alpha i > 0\}$	Goodness-of-Fit P Value	Estimate of Background	Estimate of m ± Standard Error	Goodness-of-Fit P Value	P Value (Improvement Over One-Hit)	Estimate of Background	Estimate of k ± Standard Error	Goodness-of-Fit P Value	P Value (Improvement Over One-Hit)
1	0	<.001	0	α_1,α_5	.09	.003	9.33 ± 3.07	.48	<.001	.003	28.4 ± 14.9	.48	<.001
2	0	.07	.042	$\alpha_0,\alpha_1,\alpha_2$	.49	.045	2.00 ± .53	.64	.01	.047	3.21 ± 1.40	.54	.009
3	0	<.001	0	α_3	.69	0	3.39 ± .33	.73	<.001	0	7.18 ± 1.17	>.99	<.001
4	0	.53	0	α_2	.73	0	2.01 ± .70	.85	.12	0	2.45 ± 1.18	.87	.11
5	0	.04	0	α_2	.57	0	2.00 ± .38	.63	.003	0	2.80 ± .81	.72	.003
6	.035	.03	.035	α_0,α_1	.03	0	.44 ± .12	.56	.002	0	.41 ± .002	.32	002
7	0	.99	0	α_1,α_2	.94	0	1.02 ± .26	.96	.94	0	1.02 ± .31	.96	.95
8	0	<.001	0	α_6	.13	0	6.06 ± .67	.22	<.001	0	27.57 ± 5.94	.65	<.001
9	0	.32	0	$\alpha_1,\alpha_2,\alpha_5$	.77	.009	1.69 ± .67	.81	.04	.025	1.79 ± .49	.89	.04
10	0	.33	0	α_4	.72	0	4.72 ± 2.62	>.99	.04	0	9.16 ± 7.99	>.99	.04
11	.012	<.001	.021	$\alpha_0,\alpha_3,\alpha_4$	.71	.021	3.30 ± .43	.80	<.001	.022	8.23 ± 2.09	.93	<.001
12	.098	.07	.107	$\alpha_0,\alpha_1,\alpha_2$	.36	.107	1.66 ± .35	.44	.03	.107	2.28 ± .79	.55	.02
13	.034	.16	.045	$\alpha_0,\alpha_1,\alpha_2$	.47	.049	1.44 ± .27	.22	.10	.050	1.68 ± .46	.19	.12
14	.079	.78	.086	$\alpha_0,\alpha_1,\alpha_2$	.64	.087	1.35 ± 1.05	.62	.67	.088	1.60 ± 1.78	.62	.64

TABLE 4
ESTIMATED VIRTUAL SAFE DOSE (VSD) FOR FOUR MODELS FOR FOURTEEN SUBSTANCES

Substance	Dose Unit	Estimated VSD at Risk Level 10^{-4}				Estimated VSD at Risk Level 10^{-6}			
		One-Hit	Armitage-Doll	Weibull	Multi-Hit	One-Hit	Armitage-Doll	Weibull	Multi-Hit
1	% in diet	2.0×10^{-3}	1.9×10^{-2}	.85	1.0	2.0×10^{-5}	1.9×10^{-4}	.52	.80
2	ppb.	3.4×10^{-3}	7.6×10^{-2}	.40	1.2	3.4×10^{-5}	7.9×10^{-4}	4.0×10^{-2}	.28
3	mg./kg.	4.5×10^{-3}	1.6	2.3	4.7	4.5×10^{-5}	.35	.59	2.3
4	mg./kg.	5.2×10^{-4}	1.6×10^{-2}	1.7×10^{-2}	2.5×10^{-2}	5.2×10^{-6}	1.6×10^{-3}	1.7×10^{-3}	3.8×10^{-3}
5	ppm.	3.2×10^{-3}	.19	.19	.41	3.2×10^{-5}	1.9×10^{-2}	1.9×10^{-2}	7.7×10^{-2}
6	ppm.	2.0	2.0	7.4×10^{-5}	3.0×10^{-5}	2.0×10^{-2}	2.0×10^{-2}	2.1×10^{-9}	3.9×10^{-10}
7	mg./kg.	2.1×10^{-2}	2.2×10^{-2}	2.4×10^{-2}	2.4×10^{-2}	2.1×10^{-4}	2.2×10^{-4}	2.6×10^{-4}	2.6×10^{-4}
8	ng.	8.4×10^{-6}	9.1×10^{-3}	9.2×10^{-3}	1.7×10^{-2}	8.4×10^{-8}	4.2×10^{-3}	4.3×10^{-3}	1.3×10^{-2}
9	No. of 6 hr. exposures by inhalation of 100 ppb	1.6×10^{-2}	4.0×10^{-2}	47	.48	1.6×10^{-4}	4.0×10^{-4}	3.1×10^{-2}	3.7×10^{-2}
10	% in diet	4.3×10^{-3}	1.1	1.4	2.0	4.3×10^{-5}	.33	.53	1.1
11	ppm.	5.5×10^{-2}	20.8	24.4	63.0	5.5×10^{-4}	4.5	6.0	33.5
12	ppm.	5.7×10^{-4}	2.2×10^{-3}	1.8×10^{-2}	5.1×10^{-2}	5.7×10^{-6}	2.2×10^{-5}	1.2×10^{-3}	6.7×10^{-3}
13	ppm.	2.8×10^{-2}	6.4×10^{-2}	.41	.76	2.8×10^{-4}	6.4×10^{-4}	1.7×10^{-2}	4.9×10^{-2}
14	% in diet	3.7×10^{-3}	5.7×10^{-3}	3.2×10^{-2}	6.7×10^{-2}	3.7×10^{-5}	5.7×10^{-5}	1.1×10^{-3}	3.8×10^{-3}

tihit models over the one-hit model. Table 4 presents the estimates of VSD for 10^{-4} and 10^{-6} using each of the four models. These VSD's have been calculated for each model by taking $P(D_0) - P(0) = 10^{-4}$ or 10^{-6}, since $P(D_0) - P(0)$ represents the additional risk due to the added dose D_0.

To illustrate the reading of the tables, take, for example, substance 11, ethylenethiourea. The experimental animal is the rat and the pathologic endpoint is thyroid carcinoma. The dose is measured in ppm in the diet. Table 3 shows that each of the four models estimates a small background response rate varying from .012 to .022. Furthermore, Table 3 shows that each of the three models—the Armitage-Doll model, the Weibull model and the multi-hit model—has an acceptable goodness-of-fit p value (.71, .80 and .93, respectively) in the experimental range. For example, assuming the Weibull model to be correct, the probability of seeing experimental data which fits worse than that obtained for this experiment is approximately .80, indicating a high degree of fit. However, in this case the one-hit model has a goodness-of-fit p value $< .001$ indicating that this model is a poor fit to the data. Also in Table 3, the p value for the improvement of fit for the Weibull model over the one-hit model is shown to be $< .001$. Thus, clearly the Weibull model provides a statistically significantly better fit to these data than does the one-hit model using conventional p values of .01 or .05. The goodness-of-fit p values for the one-hit, Weibull and gamma multi-hit model are based on usual chi-squared tests while that for the Armitage-Doll model is based on the simulation procedure described in Crump et al. (1977). Likewise, the p-values for the improvement of fit for the Weibull and multihit models over the one-hit model are based on likelihood ratio procedures which are not available for the Armitage-Doll model under general assumptions. (See the Appendix in Crump et al. 1977).

Finally, Table 4 presents the VSD calculations for the four models at risk levels of 10^{-4} and 10^{-6}. Looking again at substance 11, ethylenethiourea, note that the range is 5.5×10^{-2} to 63.0 ppm at a risk level 10^{-4} and 5.5×10^{-4} to 33.5 ppm at a risk level 10^{-6}. Note that in this case the one-hit model because of the imposed low-dose linearity yields a much smaller VSD than the other three better fitting models which

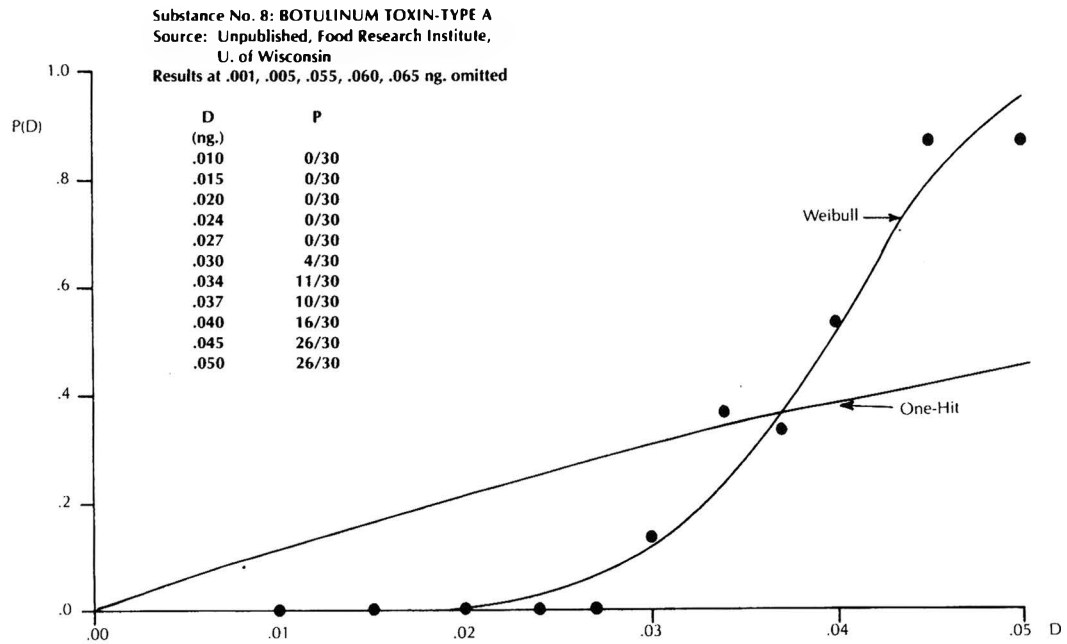
Substance No. 8: BOTULINUM TOXIN-TYPE A
Source: Unpublished, Food Research Institute,
U. of Wisconsin
Results at .001, .005, .055, .060, .065 ng. omitted
D (ng.) P
.010 0/30
.015 0/30
.020 0/30
.024 0/30
.027 0/30
.030 4/30
.034 11/30
.037 10/30
.040 16/30
.045 26/30
.050 26/30
P(D)
1.0
.8
.6
.4
.2
.0
.00
.01
.02
.03
.04
.05
D
Weibull
One-Hit

Fig. 1a

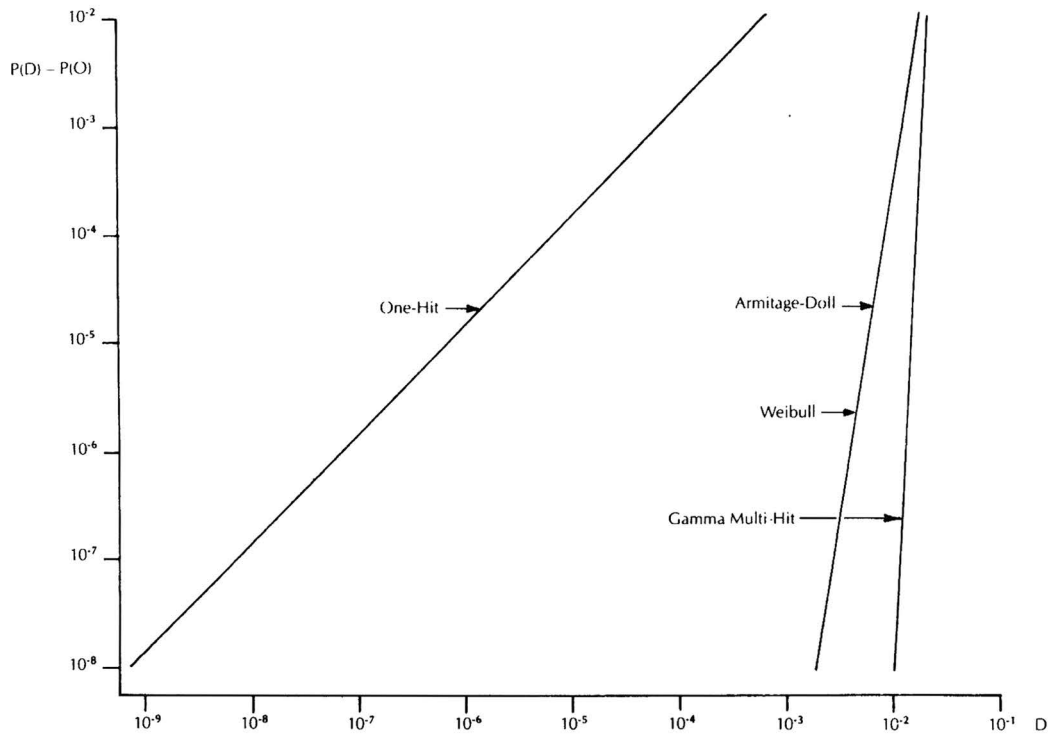
P(D) – P(O)
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
D
One-Hit
Armitage-Doll
Weibull
Gamma Multi-Hit

Fig. 1b

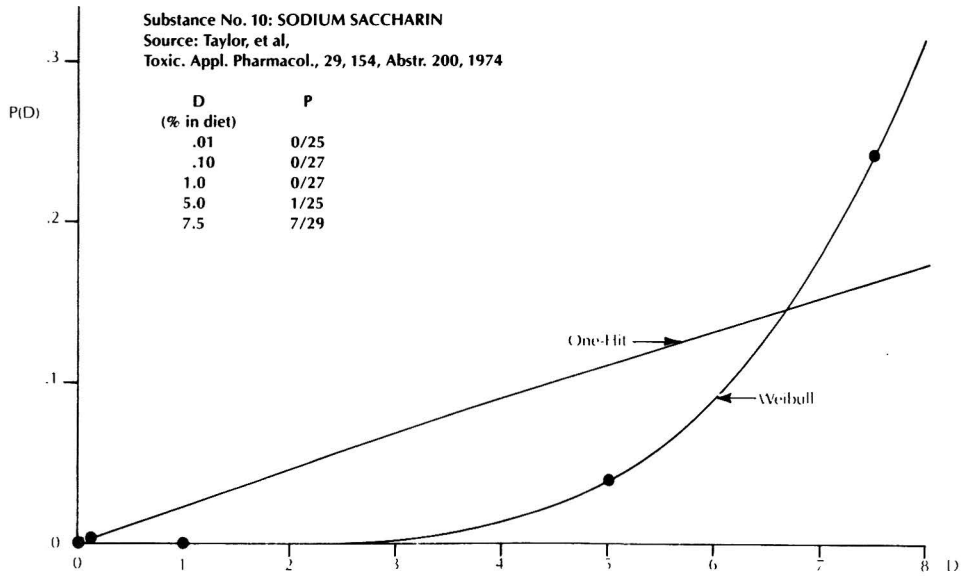
Substance No. 10: SODIUM SACCHARIN
Source: Taylor, et al,
Toxic. Appl. Pharmacol., 29, 154, Abstr. 200, 1974
D (% in diet) P
.01 0/25
.10 0/27
1.0 0/27
5.0 1/25
7.5 7/29
P(D)
.3
.2
.1
0
One-Hit
Weibull
0 1 2 3 4 5 6 7 8 D

Fig. 2a

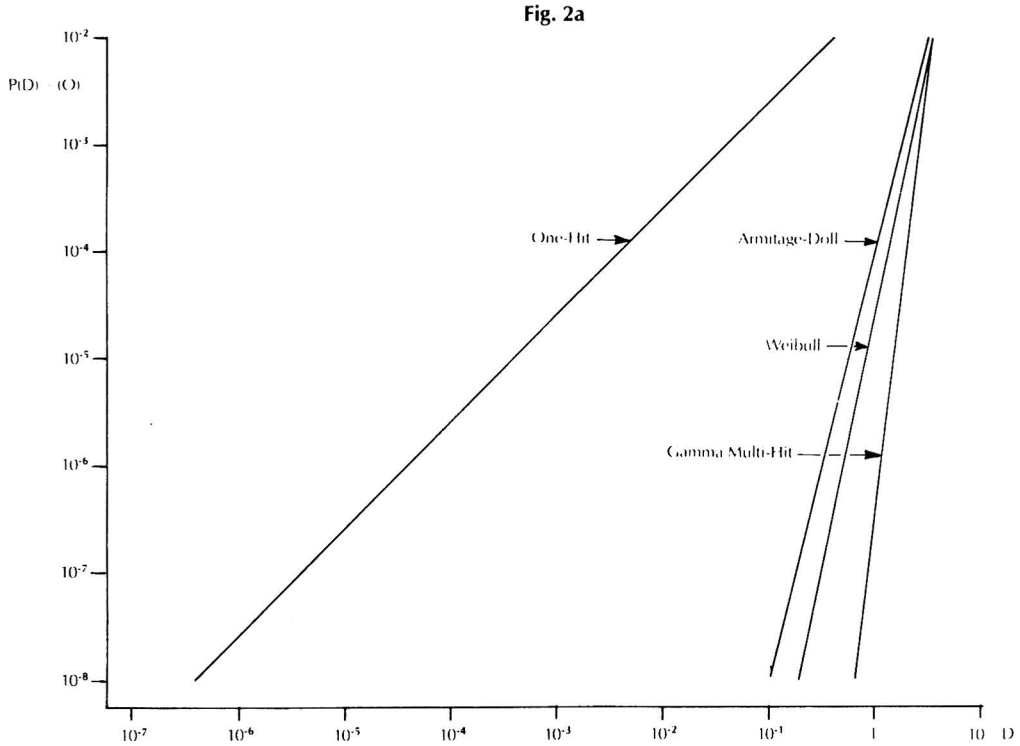
P(D)
One-Hit
Armitage-Doll
Weibull
Gamma Multi-Hit
10 D

Fig. 2b

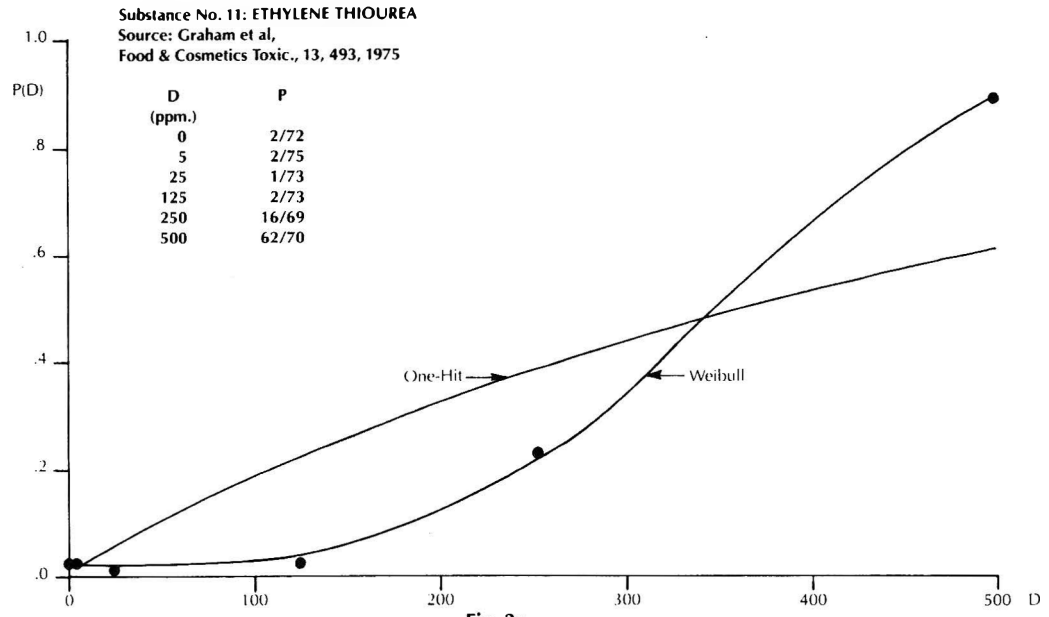
Substance No. 11: ETHYLENE THIOUREA
Source: Graham et al,
Food & Cosmetics Toxic., 13, 493, 1975
D (ppm.) P
0 2/72
5 2/75
25 1/73
125 2/73
250 16/69
500 62/70
P(D)
1.0
.8
.6
.4
.2
.0
One-Hit
Weibull
0
100
200
300
400
500
D

Fig. 3a

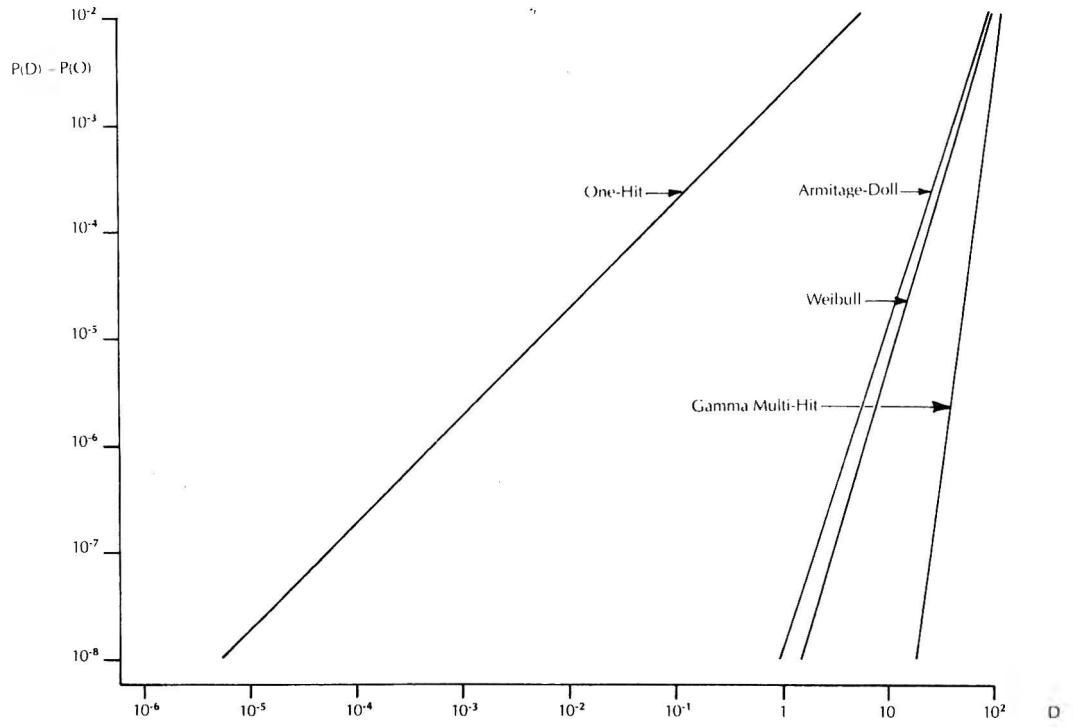
P(D) - P(O)
10-2
10-3
10-4
10-5
10-6
10-7
10-8
One-Hit
Armitage-Doll
Weibull
Gamma Multi-Hit
10-6
10-5
10-4
10-3
10-2
10-1
1
10
10²
D

Fig. 3b

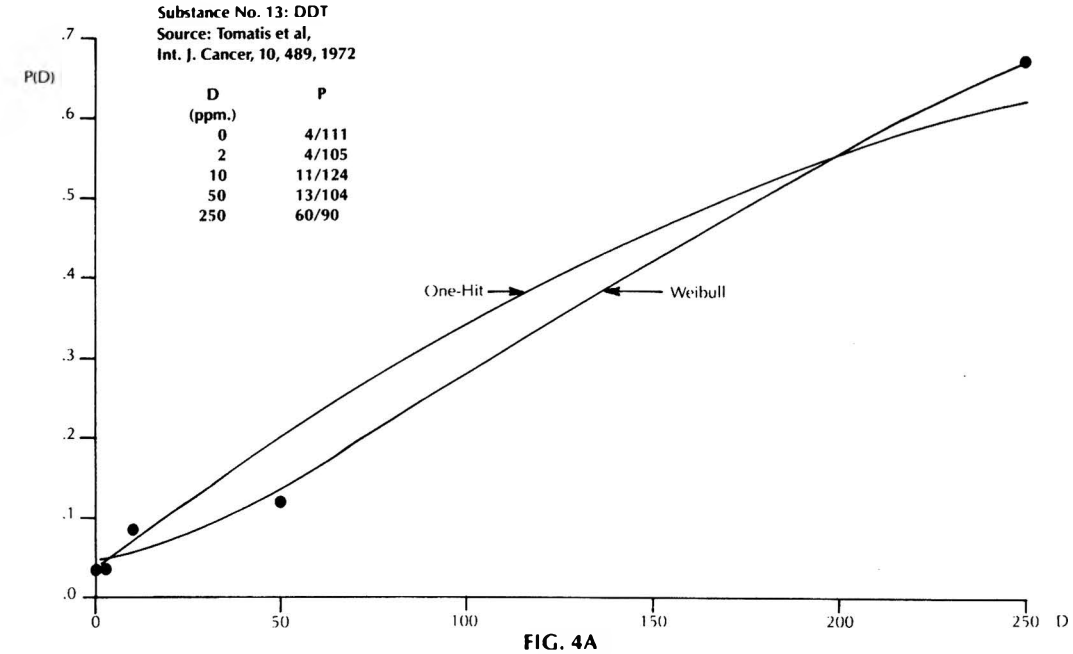
Substance No. 13: DDT
Source: Tomatis et al,
Int. J. Cancer, 10, 489, 1972
D (ppm.) P
0 4/111
2 4/105
10 11/124
50 13/104
250 60/90
P(D)
.7
.6
.5
.4
.3
.2
.1
.0
One-Hit
Weibull
0
50
100
150
200
250
D

FIG. 4A

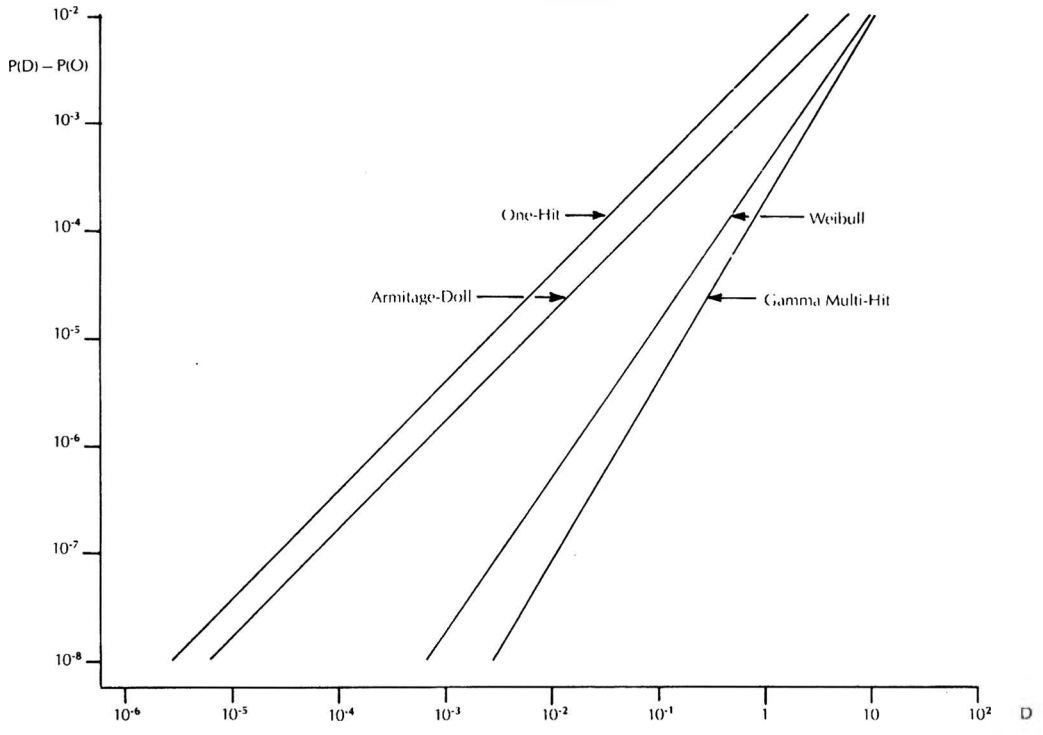
P(D) – P(O)
10-2
10-3
10-4
10-5
10-6
10-7
10-8
One-Hit
Weibull
Armitage-Doll
Gamma Multi-Hit
10-6
10-5
10-4
10-3
10-2
10-1
1
10
10²
D

Fig. 4b

allow for low-dose non-linearity, but do not impose it. The non-linearity in the observed data for ethylenethiourea is exhibited in the VSDs in Table 4 as well as in the estimates of the α_i's for the Armitage-Doll model ($\alpha_1 = 0$) in Table 3 and the estimates of m(3.30) and k(8.23) for the Weibull and multi-hit models, respectively.

Hexachlorobenzene, substance 7, provides an example where all the models are in close agreement. In that case all the models fit the data equally well in Table 3. The VSD's given in Table 4 for these four models are in a narrow range from 2.1×10^{-4} to 2.6×10^{-4} mg per kg at a risk level of 10^{-6}. The close agreement on linearity in the low dose range is indicated by the parameter estimates in Table 3 with $\alpha_1 > 0$ in the Armitage-Doll model, m = 1.02 in the Weibull model and k = 1.02 in the gamma multi-hit model.

The results in Tables 3 and 4 are also illustrated graphically on four substances: botulinum toxin (No. 8), sodium saccharin (No. 10), ethylenethiourea (no. 11), and DDT (no. 13). The goodness of fit of the Weibull and one-hit models is depicted in Figures 1a to 4a with the low dose VSD's for all four models shown in Figures 1b to 4b. (The dose response curves in the observable range for the Armitage-Doll and gamma multi-hit models are similar to those for the Weibull model shown in Figures 1a to 4a). Note that for the cases of botulinum toxin, sodium saccharin and ethylenethiourea the plots show good fits with the Weibull model and poor fits with the one-hit model. This is reflected for the botulinum and ethylenethiourea examples by the goodness-of-fit statistics of Table 4, but not in the case of sodium saccharin. The reason for this is that for sodium saccharin the sample sizes are relatively small with only two positive response points and thus the power to detect the lack of fit in the case of the one-hit model is limited. Note, however, that the Weibull and gamma multi-hit models provide significantly better fits in this case ($p < .04$) than does the one-hit model.

The example of DDT shows a case in which the Weibull and one-hit models fit the data acceptably well and the range of VSD's is not as severe as in the other three cases plotted. Such agreement or disagreement is reflected in the plots of log-response versus log-dose in the low-dose range for all four models in Figures 1 to 4.

Clearly, there are some substantial differences among these four models. Generally, the one-hit model gives the lowest VSD, the Armitage-Doll the second lowest, the Weibull the second highest, and the k-hit model gives the highest. An obvious exception is substance 6 (vinyl chloride), where the dose-response function is concave over the observed dose region and the usual order among the four models is reversed. In the case of vinyl chloride the question of whether the high doses as administered are the true "effective" dose to the target organ because of metabolic activation has been discussed by Gehring et al. (1978) and Van Ryzin and Rai (1980). If one were to apply the models to only the first four dose or the "effective" dose levels the disparity between the models is considerably reduced. For details see Van Ryzin and Rai (1980).

Recommendations for Risk Assessment Methods

6. There are three related but logically distinct elements involved in the choice of method: the choice of a model, the choice of an extrapolation procedure and a societal judgment as to the acceptable risks in light of the benefits or lack thereof of the agent under consideration.

a. *The choice of the model.* The models that currently seem usable for low-dose extrapolation are the probit model, the one-hit model, the k-hit model, the Armitage-Doll model, and the Weibull model. The statistico-pharmacokinetic model, which is in many respects the most biologically realistic of all, is excluded because it appears to require quantitative information on metabolic pathways that will often not be available. The one-hit model is really a special case of the Armitage-Doll model ($\alpha_1 > 0$, $\alpha_i = 0$ for $i \geqq 2$), the k-hit model (k = 1) and the Weibull model (m = 1).

A four dose-level experimental design is recommended that will lead to responses permitting the estimation of the multiple parameters of the Armitage-Doll, k-hit or Weibull models and the background (control) response as well, more often than designs now in use.

In contrast with the proposed 5-dose design in this report, the protocol used in the National Cancer Institute Carcinogenic Bioassay Program calls for one control group and two animal groups at positive dose levels. As such the data available from such an experiment will often be of little use in fitting the multiple parameter models (Armitage-Doll, k-hit, and Weibull). Detecting differences in fit to these models over the one-hit model will be virtually impossible.

If the estimated values of the parameters lead to values close to those of the one-hit model, the choice of the model will then be the one-hit model, but this choice will be governed by data rather than *a priori* considerations. Other models (probit, multi-hit, Armitage-Doll and Weibull) will describe most data sets in the 5% to 95% range equally well and the choice among them must depend on other considerations, such as the rapidity with which each approaches control response as dose approaches zero. The probabilities yielded by the probit model approaches zero faster than any power of D, the k-hit model as the k^{th} power of D, the Weibull model as the m^{th} power of D, and by the Armitage-Doll model most slowly, as the first power of D for $\alpha_1 > 0$. A major mathematical basis for this slow approach in the Armitage-Doll model, is the *ad hoc* assumption that the somatic mutation rate is of the form of $\alpha+\beta$ D, where D is dosage, α the spontaneous mutation rate (presumably often the result of exposure to physical or chemical carcinogens) and $\beta \geq 0$. This is a very strong assumption which is not supported by (a) the general lack of evidence for dose-wise additivity shown by many experiments in joint effects of two or more agents, as discussed in Section 2, or (b) experiments in which the effects of one tumor inducing agent inhibits those of another, or (c) experiments, such as those of Hsie et al. (1978) with methanesulfonate in Chinese hamster ovary cells, in which an *in vitro* linear dose-response curve for mutation disappears when performed *in vivo*, in agreement with the view that whole body defense mechanisms impair linearity.

Some agents which are carcinogenic at high dose levels have little or no observed effect at low dose levels, in apparent disagreement with the linear assumption (See, however, National Academy of Sciences, 1977 p. 40). Thus, nitrilotriacetic acid (NTA), which led to kidney tumors in 12 out of 48 rats when fed at 2% in the diet had an incidence of 2 out of 91 at 1.5%, 1 out of 91 at 0.75%, 0 out of 48 at both 0.2% and 0.02% and 0 out of 127 in control rats. Other high dose carcinogens, considered in the previous section, which show little linearity are sodium saccharin, and ethylenethiourea.

Results of the ED_{01} experiment of the National Center for Toxicologic Research (NCTR) given in Littlefield, et al. (1979) show a highly non-linear dose response incidence of bladder tumors in mice sacrificed at 24 months. A recent analysis of that data by Dr. Frank Carlborg (1980), showed a lack of fit p value of less than .001 for the one-hit model.

Results of this type cannot, in principle, disprove the existence of a small but non-zero linearity which eludes detection in experiments of finite size. In this sense, both the concepts of a threshold and of low-dose linearity are opposite sides of the same non-operational coin, neither being generally capable, even in principle, of direct experimental proof or disproof. Mantel's observation and Crump's at least partial agreement b (Mantel, 1978) that the additivity assumption leading to low-dose linearity, "is not a scientifically established fact," puts the model in its proper perspective—as a hypothesis that may perhaps lead to further experiments or provide alternative explanations for already existing data, but lacking detailed experimental support.

Some carcinogens yield dose-response curves that are more linear or sublinear in the observed dose range, such as vinyl chloride and 2-AAF induced liver tumors in the NCTR

results, and for such agents no assumption other than low-dose linearity seems possible. The above argument is critical, not of low-dose linearity when it is justified by the data, but of its assumed general or near-general applicability no matter what the data. What is required is a procedure which assumes low-dose linearity when data in the observable range suggest this, but rejects the assumption when the shape of the curve suggests that the high doses have saturated the repair, protective or other mechanisms by which multi-cell organisms cope with low doses—without, however, assuming the existence of unobservable population thresholds. The k-hit, Armitage-Doll, and Weibull models all have this feature when one uses them to obtain or point estimates of risk. For this purpose, we recommend the use of these three models. There may be special circumstances under which biological considerations not reflected in the observed dose-response curve would suggest low-dose linearity. Nothing that has been said is meant to preclude giving weight to such considerations.

b. *Choice of extrapolation procedure.* Many of the extrapolation procedures that have been proposed appear to incorporate value judgments in ways that are not always easily identifiable, particularly in the choice of so-called conservative procedures, such as the use of slopes shallower than shown by the data in the Mantel-Bryan procedure and the use of upper confidence limits to response or lower confidence limits on VSD in the Mantel-Bryan and Crump et al. procedures as well as the Van Ryzin and Rai (1980) conservative procedure for the k-hit model. Although the value judgment involved in the use of conservative risk assessments may seem appropriate in the light of the many scientific unknowns involved, once formalized as a specific mathematical procedure it escapes the control of the decision-maker and can lead to undesirable and unsound results by distorting the balance between risk and benefit. We therefore recommend the separation of the mathematical and societal aspects of the problems, with the extrapolation procedure chosen to provide "best estimates" or point estimates of risk. The dubious meaning of confidence limits when the model is incorrect which is the case in the absence of firm evidence of the biological appropriateness of any one model, provides another reason for confining the extrapolation procedure to best estimates with a variety of models.

As has been seen from Tables 1 to 4, the low-dose extrapolated risk estimates are highly model dependent. Because of this inexactness of the behavior of the models in the low-dose range, plus the fact that they cannot be firmly justified on either statistical (goodness-of-fit, say) or biological grounds, the choice of how one does the extrapolation is primarily a matter of judgment. However, since regulatory decisions must be made, these should be done based on general scientific considerations. This includes picking one of the models which incorporate low-dose linearity as well as nonlinearity (k-hit, Weibull and Armitage-Doll) together with an extrapolation procedure with the desired conservativeness. Under certain circumstances outlined above a conservative procedure imposing low-dose linearity may be warranted.

c. *Choice of Societal Risk Level.* Having outlined the choice of model and the method of extrapolation to be used based on principles of sound scientific judgment, both biological and statistical, the next step is to choose the risk level P_0 to which one extrapolates. We have attempted to give procedures which separate statistical and biological judgment from societal judgment. This separation implies only that the societal value judgments should appear at a later stage in the decision tree and not that they should be disregarded. A major element in this judgment will be the choice of an allowable level of risk, P_0. The choice of $P_0 = 10^{-6}$ by the Commissioner of the Food and Drug Administration was made after much discussion. It nevertheless seems to us that its value should not be fixed in advance for all agents and that its choice must depend on the value to society of the agent involved. Thus, the risk level that

society may be willing to tolerate for the only remaining non-nutritive sweetener saccharin, may well be greater than that of an agent used for cosmetic purposes in food.

Other Important Aspects of Low-Dose Risk Assessment

7. Even after a mathematical model has been agreed upon, a number of other important questions must be addressed. These include:

a. *Combination of Results For Two or More Species.* It is recommended that the chronic test be carried out on two species. The low-dose risk assessment of results in each of these species need not be the same. It is by no means uncommon to find agents that are carcinogenic in mice but not in rats, or the reverse. A conservative procedure is to use the results in the most sensitive species, but, as we have argued, this involves a societal judgment. Knowledge of intermediate metabolism in the species and in man may suggest which is the more appropriate, but in the absence of such information, informed scientific opinion would favor use of the most sensitive species even though the averaging of species results seems more consistent with the idea of best estimates considered in Section 6 of this Chapter.

b. *Combination of Separate Studies on the Same Species.* Independently conducted bioassays on the same species should be analyzed concurrently or in combination to the extent that the experiments are biologically and statistically compatible. With complete compatibility, several experiments can be analyzed as one. With the Weibull model, it is also easy to outline an analysis of several experiments which are only partially compatible. For example, it is sometimes possible to allow the intercept α and the slope β to vary among the experiments (or between the sexes within an experiment), but to have one common value of the exponent m. Every effort should be made to estimate the parameter m with as little error as possible, because it is critical in the suggested method in Section 4 and ultimately so important for the estimated VSD.

c. *Interspecies Extrapolation.* The basis of extrapolation from doses administered to test animals to the corresponding human levels of exposure is an important consideration. The following are the most frequently-used procedures.

Option 1. The administered dose of test substance is expressed as a fraction of the total diet fed to the test animals, that is as ppm or ppb of the experimental diet as fed on a dry weight basis. The assumption is made that adult human intake can then be expressed in terms of ppm or ppb on a similar basis, generally considered to be 600-700 g per day. [Fed. Reg. *44*, 17070 (1979)].

Option 2. The administered dose is expressed in terms of mg per kg body weight of test animal. Human exposure is then considered on the same basis of body weight.

Option 3. Dosage in terms of mg/kg is converted to mg/sq. m. of body surface area. Human exposure is also expressed in terms of mg/sq. m. of body surface area.

The fallacies inherent in the choice of Option 1 or Option 3 are discussed by Oser (1979). Briefly, Option 1 makes no allowance for the substantial differences in daily food consumption (particularly by rodents) during rapid growth in the post-weaning phase, as compared with more gradual weight gain in early adult or fully adult life. The consequence is a huge excess exposure, from weaning to adolescence, over the intended level of consumption of the test substance.

Option 3 is also based on fallacious assumptions. Klippel (1979) has drawn attention to the fact that currently-used procedures for arriving at body "surface area" do not represent anything resembling true skin area. In spite of such errors, Option 3 apparently finds satisfactory application in cancer chemotherapy. That is no reason for using it in the case of food components.

A comparison of extrapolations from mouse or other animal species to man (Table 6) by Paget (1965) reveals the implications of the approaches based on body weight as opposed to surface area. The ratio of the two results in the

case of man is out of all proportion to the ratios in laboratory animals. With long experience of the value of extrapolation on a body weight basis, we recognize this as the most satisfactory procedure and will use it henceforth for all calculations in this discussion.

TABLE 5

A DOSE OF 100 MG. PER KG. IN THE MOUSE EXTRAPOLATED TO OTHER SPECIES BY WEIGHT AND BY SURFACE AREA

	Weight (grams)	Surface area* (sq. cm.)	A Absolute dose by weight (mg.)	B Absolute surface area (mg.)	Ratio A/B
Mouse	20	46.4	2	2	1.0
Rat	200	324.8	20	14	1.43
Guinea pig	400	564.5	40	24.3	1.65
Rabbit	1,500	1,272.0	150	54.8	2.74
Cat	2,000	1,381.0	200	59.5	3.46
Monkey	4,000	2,975.0	400	128.2	3.12
Dog	12,000	5,766.0	1,200	248.5	4.82
Man	70,000	18,000.0	7,000	775.8	9.8

*Surface area (except in case of man) calculated from formula:

$$\text{Surface Area } (cm^2) = k(W^{2/3})$$

where k is a constant for each species and W is body weight (values of k and surface area of man taken from Spector[4]).

d. *Accounting for Variations in Human Ingestion.* Although the risk at an average use level may be acceptably low, some individuals may consume well above this level and be subject to higher, and perhaps unacceptably higher, risk. This is the so-called glutton problem and the question of how far up on the gluttony scale protection should be provided must be faced. We recommend the principle of evaluating risk at the upper 90th percentage point on the consumption scale.

e. *Multiple Responses.* Some agents may lead to more than one type of undesirable response, e.g. liver and bladder tumors, as in the case of 2-AAF. The VSD for risk for each organ alone may be higher than the VSD for the combined risk, and when the individual responses are considered equally undesirable, the combined risk should also be determined.

Dose-response curves for each response separately lead to separate estimates of risk at the different dose levels and these may be added to obtained estimates of the combined risk, from which a combined VSD can be determined. In principle this should be done separately for each of the mutually exclusive combinations, e.g. response A and no response B, response B and no response A, and both A and B, but in practice the occurrence of joint responses in the same laboratory animal may not be that common.

f. *Concurrent and Historic Controls.* It is good, although far from universal, laboratory practice to allocate each batch of animals at random among the various dosage groups, including the controls. The statistical procedures used to fit the model implicitly assume that this was done. These procedures can therefore give considerable weight to one or two positive responses in the control animals in the low dose extrapolation when a two-parameter dose response curve is used, whereas historic variation from batch to batch in control response might suggest that such responses should not be given much weight. In recent years statistical procedures have been developed to use information on the historic control response and its batch to batch variation (Pocock, 1976). They have not been applied to the fitting of dose-response curves and when the implicit randomization assumptions are strictly satisfied might have little to contribute. But it is always difficult to be sure that all important sources of variation have in fact been randomized among the dosage groups even in well-run laboratories. When the historic variation is greater than that assumed by existing statistical procedures, the uncertainty of the risk assessments would be greater than they indicate. The systematic development by individual laboratories of information on variation in control responses and its incorporation into fitting procedures, with a view to comparing results

calculated using historic and concurrent controls, would therefore be worthwhile.

g. *Confidence Limits versus Best Estimates.* There is one strong argument for using a lower confidence limit for the VSD rather than the statistical best estimate of the VSD when using the k-hit, Armitage-Doll or Weibull models. With data from a small or poorly designed experiment, the shape parameter k for the k-hit model or the exponent parameter m for the Weibull model would be estimated with considerable uncertainty or error. Since the low-dose extrapolation estimates are sensitive to the values of these parameter estimates, the final estimate of VSD is suspect. In the Armitage-Doll model the estimate of the coefficients α_1, α_2, etc. control the low-dose behavior and these may be estimated as zero with a high degree of uncertainty from a small or poorly designed experiment. To be safe in the presence of such uncertainty, it has been suggested that using a lower confidence limit for the VSD be used rather than the best estimate of VSD.

We believe that doing the VSD calculations at various risk levels for all four models together with their goodness-of-fit statistics and plots gives enough statistical information for risk assessment decisions. Calculations of confidence limits in addition will shed little additional information since they too are highly model dependent.

Summary

The choice of mathematical procedures for low-dose extrapolations has no firm biological basis and must to some extent be arbitrary. But the chief alternative to low-dose extrapolation is the setting of zero tolerances for any substance that is harmful at sufficiently high doses, i.e., nearly everything. But zero tolerances are not reasonable alternatives for many agents which have clear benefits for man. Thus, risk assessment is unavoidable and must, implicitly or explicitly, involve a balancing of risk and benefit. We recommend the use of calculations of VSD's from the four models—the one-hit model, the Armitage-Doll model, the Weibull model, and the gamma multi-hit model as inputs into the decision procedures. These calculations should be done for a variety of risk levels in the range of societal concern. Which estimate should be used for each agent by the decision-making authorities should then use the principles outlined above. The choice of estimates made should reflect the use of the more flexible models (Armitage-Doll, Weibull, and multi-hit) when they fit better and seem appropriate biologically. If a conservative procedure is warranted based on the biological considerations outlined earlier, then the use of the one-hit model or some other low-dose linear extrapolation procedure seems justified. However, blind use of low-dose linear extrapolations or the conservative one-hit model appears to us to be scientifically indefensible.

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REVIEWS OF RECENT PUBLICATIONS

Glutamic Acid: Advances in Biochemistry and Physiology. Edited by L. J. Filer, Jr., S. Garattini, M. R. Kare, W. A. Reynolds and R. J. Wurtman. Raven Press, New York, 1979. pp. xiv + 400. £20.75.

Published in the Monographs of the Mario Negri Institute of Pharmacological Research series, this book is based on the proceedings of an international symposium on the biochemistry and physiology of glutamic acid held in 1978. Balanced reports are presented by leading scientists in various fields of glutamate research. The contributors include J. W. Olney, who claims that high doses of glutamate can produce neurotoxic effects in primates and who is the main protagonist in the current debate over the acceptability of aspartame as an artificial sweetener in the USA.

It is a compact volume, yet it contains an impressive amount of useful information, particularly for those who are interested in glutamate from the standpoint of food safety and nutrition. The first of its five sections deals with the sensory and dietary aspects of glutamate occurring naturally in foods or added commercially as a flavour enhancer. Glutamic acid's role in intermediary metabolism, and interspecies differences in the metabolism of glutamate from exogenous sources are reviewed in the second section. The third is concerned with access of circulating glutamate to the brain, its synthesis and metabolism within the brain and a putative role for glutamate as an excitatory neurotransmitter. This leads on well to the fourth section, which discusses safety evaluation studies on glutamate and its use in neurotoxicological research. Animal toxicity data are reviewed, and particular attention is paid to the conflicting evidence on the production of hypothalamic lesions by high doses in neonatal monkeys. The hormonal, behavioural and reproductive effects in laboratory animals and the interspecies variations in sensitivity to glutamate are also discussed. Notwithstanding the continuing controversy about some aspects of these studies, extrapolation from the kinetics of orally administered glutamate in newborn mice (the species most sensitive to neurotoxic effects) suggests that there is a considerable safety margin between even unpalatable doses and the doses likely to lead to neuronal damage in human adults. The final section of the book covers clinical aspects, including population surveys connected with the use of glutamate in food.

Bailey's Industrial Oil and Fat Products. Volume 1. 4th Ed. Edited by D. Swern. John Wiley & Sons, New York & Chichester, 1979. pp. xii + 841. £28.00.

This new (fourth) edition of 'Bailey' will be welcomed by those in the food industry who have had such good service from the previous edition, which was published in 1964 (*Cited in F.C.T.* 1965, **3**, 888). For the first time, this invaluable text is being published in two volumes, which together will contain about twice as much information as the previous presentation.

Volume 1, like the third edition, is edited by Daniel Swern, and this time the chapters are contributed by M. W. Formo, E. Jungermann, F. A. Norris, and N. O. V. Sonntag. It contains ten of the 23 chapters that appeared in the third edition and those on the structure and composition of fats and oils, reactions of fats and fatty acids, fats in the diet, the composition and characteristics of individual fats and oils, soap and soap manufacture, and fat-based surfactants have been particularly revised and expanded. In addition, some degree of change is apparent in the four other chapters, which cover the physical properties of fats and fatty acids, sources, utilization and classification of oils and fats, the handling, storage and grading of oils and of oil-bearing materials, and paints, varnishes and related products.

Chapter 4, on fats in the diet, has been considerably expanded to include a lucid discussion of the effects of fats on health. A well-balanced view of the role of fats in coronary heart disease is presented, and supported by a large number of references. Other topics dealt with in this section are the toxicity of oxidized and heated fats, and the safety of additives used in fatty foods, of hydrogenated fats and of specific fatty acids (including, briefly, erucic acid). Another chapter that clearly reflects changes in the fats and oils industry during the last two decades deals exclusively with fat-based surface-active agents other than soap. It includes some discussion of non-fatty surfactants and contains an interesting section on the developments that have occurred to meet the increased concern about the effects of detergent products on the environment. These have led to the production of more readily biodegradable products.

Volume 1 is a valuable book in its own right. When the second volume is published, this fourth edition will be a very worthy replacement for its well-thumbed predecessor.

Nutritional and Safety Aspects of Food Processing. Edited by S. R. Tannenbaum. Marcel Dekker, Inc., New York, 1979. pp. ix + 448. Sw.fr. 100.00.

In this neat presentation, the sixth book in a series of food science monographs edited by O. R. Fennema, an integrated approach has been used to present the chemical, biochemical, nutritional and toxicological aspects of food processing. Approximately half of the book is devoted to basic nutrition and a consideration of the 'plus' and 'minus' effects of food processing on individual nutrients. An interesting chapter deals with mathematical calculations for predicting nutrient losses and determining optimum processing conditions. Food fortification, preservatives and immunological aspects of food safety are also examined.

and there is an extensive chapter on residues of agricultural chemicals in foods and the ways in which they may be affected by processing techniques.

Each of the twelve concise chapters (written by some 16 contributors) is subdivided by topic and is complete with an introduction, summary and extensive reference list. Full subject and author indexes are also provided.

The monograph on antinutritional and toxic substances discusses in a logical manner both naturally occurring toxicants and accidental contaminants. A brief general survey of currently recognized problem areas, with detailed information on the more important aspects, includes sections on bacterial toxins and mycotoxins, natural constituents of foodstuffs of plant and animal origin and intentional and unintentional additives. An interesting table summarizes the essential features of toxic constituents in plant foodstuffs, revealing the chemical nature and major toxic effects of those present in commonly eaten foods. These include the goitrogenic thioglycosides found, for example, in cabbage, turnips and rapeseed, and the haemagglutinins, which are proteins of high molecular weight present in some types of bean and in lentils and peas. Haemagglutinins may impair growth and food utilization and have been shown to cause agglutination of erythrocytes and mitogenic activity in cell cultures *in vitro*. Other relevant points concerning these toxicants are discussed in the text.

Although this volume will be of interest primarily to the food scientist, the inter-disciplinary approach used throughout means that it will be of use also to the manufacturer, the nutritionist and the toxicologist in linking the biological sciences with the principles and practices of modern-day food processing. Whilst of value as a general reference text, the book can equally provide a useful introduction to the literature as a prelude to in-depth studies on specific aspects of food safety.

Chemical Carcinogenesis. Edited by P. Brooks. British Medical Bulletin, Vol. 36, no. 1. Medical Department, The British Council, London, 1980. pp. 104. £6.00.

The first issue of the British Medical Bulletin to be devoted to chemical carcinogenesis was published in 1947, and the latest of several successors has now appeared. In it, an attempt has been made to give some structure to a varied collection of articles, beginning with the history and future of chemical carcinogenesis and continuing with more specific topics, ranging from the molecular biology level through to epidemiology. There is inevitably some overlap and discontinuity between chapters but on the whole the attempt has been successful.

The introductory chapter, by E. Boyland, is followed by the now traditional topics of carcinogen metabolism, interaction with DNA, and DNA repair, presented respectively by P. Sims, P. D. Lawley and J. J. Roberts, three respected figures in these fields. Following these, J. P. Roscoe describes a promising *in vivo/in vitro* system for investigating the cellular changes that lead to malignancy during the latent period of carcinogenesis. After this point, the logical sequence of chapters is overcome in some measure by the diversity of the topics, which include bladder cancer, the biological effects and metabolism of aflatoxin B_1, asbestos carcinogenicity, bracken fern and papilloma virus in bovine alimentary cancer, bacterial metabolism and human carcinogenesis, and interactions between carcinogens and proteins.

Dealing with an area in the mainstream of current research, S. Venitt reviews bacterial mutagenesis, its history, the assays at present in use and their validity as predictors of carcinogenic potential. These and other short-term tests for carcinogencity are not without their problems, however, and as J. Ashby and J. A. Styles clearly demonstrate, many of the shortcomings centre on the fallibility of the metabolizing systems used.

No modern review of chemical carcinogenesis would be complete without a contribution from the expanding discipline of immunology, and M. J. Embleton and R. W. Baldwin indicate some of the reasons for the failure of immunology to make the impact that had been hoped for in the treatment of cancer. Finally, a chapter by M. Alderson on the future of epidemiology stresses the importance of obtaining accurate data and developing prospective studies, and underlines the need for greater collaboration between epidemiologists and laboratory scientists.

As may be anticipated from the list of contributors, this volume is both authoritative and wide-ranging and so has much to offer to all readers with a genuine interest in chemical carcinogenesis.

Le Lavage Broncho-alvéolaire chez l'Homme: Bronchoalveolar Lavage in Man. Edited by G. Biserte, J. Chrétien and C. Voisin. Editions INSERM, Paris, 1979. pp. 554. Fr.fr. 80.00.

Recent studies of the pathogenesis of respiratory diseases have confirmed the importance of trying to understand the early cellular events that occur and that may initiate or amplify the development of these diseases. Bronchoalveolar lavage has developed with the advent of fibroptic bronchoscopy, and is now being used in clinical practice. The proceedings of a symposium held in Lille in February 1979 present a useful summary of the biochemical and immunological data that have been obtained by these techniques. The three main topics discussed at the symposium were the biochemistry of bronchoalveolar-lavage fluids, the characteristics and functions of bronchoalveolar cells obtained by lavage and the application of this technique to clinical studies.

Several contributors discussed the glycoproteins found in lavage fluids. These include fibronectin and other secretory glycoproteins, such as alveolyn which is found in respiratory epithelial cells. These proteins bind collagen and hyaluronic acid and may be necessary for alveolar stability and defence. The amounts of immunoglobulins such as IgA, IgG and IgM in the lavage fluid were found to vary in patients with benign or malignant diseases. The difficulty of standardizing the amounts of protein and lipids obtained in bronchoalveolar fluid was discussed, as the amount of fluid recovered varied according to the clinical state of the patient. It was suggested that potassium, which is stringently regulated in the alveolar spaces, should be used as a reference substance.

On the whole the work discussed in this section demonstrated that it is possible to obtain a biochemical profile of the bronchoalveolar fluids. In spite of the wide individual variations that occur, differences are evident between the levels of protein, phospholipids and enzyme secretions in healthy controls and those in subjects suffering from allergic alveolitis and diffuse interstitial lung diseases. Changes in surfactant production as well as in the composition of surfactant can also be evaluated by these techniques.

The second section of the symposium was concerned with the characteristics and functional abilities of the populations of bronchoalveolar cells obtained by lavage techniques. A number of investigators discussed the normal cell biology of the alveolar macrophage, its alterations in response to changes induced by cigarette smoke and the relationship between abnormal macrophage function and the pathogenesis of lung disease. Macrophages obtained from the lungs of cigarette smokers show membrane and functional changes characteristic of stimulated macrophages and are recovered in much greater numbers than from non-smokers. An interesting observation was an increase in lung T lymphocytes with a diminished mitogen responsiveness in smokers, suggesting that cigarette smoke may impair local immune defences. Differences between the lymphocyte populations of fluids obtained from non-smokers and from patients with sarcoidosis or hypersensitivity pneumonitis indicated that lavage fluids could be useful diagnostically.

The final group of presentations was in fact concerned with clinical applications. There was general agreement that analysis of bronchoalveolar fluid from patients with interstitial lung diseases was providing information that was of value in unravelling the basic immunopathology underlying many of these diseases. The identification of certain enzymes, such as collagenase, in the fluid may help to explain alterations in collagen structure, and bronchoalveolar lavage is also important for the bacteriological study of infectious lung diseases.

The information provided in these proceedings brings together the work of groups of investigators who have experience of this technique, and thus provides a broad view of the use and interpretation of this approach and its importance to the understanding of respiratory diseases.

Cellular, Molecular, and Clinical Aspects of Allergic Disorders. Edited by S. Gupta, and R. A. Good. Comprehensive Immunology. 6. Plenum Medical Book Company, New York, 1979. pp. xix + 628. $54.00.

The sixth book in the series *Comprehensive Immunology* describes the cellular, molecular and clinical aspects of allergic disorders. The first three chapters are concerned with the cellular basis of allergic disease and the body's adjustment to allergic reactions. The structure, function and roles of mast cells and basophils, as well as the ability of these cells to synthesize and release both histamine and low-molecular mediators of immediate hypersensitivity reactions, are discussed with great clarity. Although the roles of basophils and mast cells in delayed-onset reactions, including reactions to many protein antigens, have been recognized only recently, the review of this topic indicates the rapidity with which evidence for the participation of these cells in such reactions is accumulating. The function of eosinophils is less well defined at present, but these cells are known to play an important role in immunoglobulin E-mediated hypersensitivity states such as anaphylaxis, asthma, allergic rhinitis and some drug allergies.

Having thus reviewed the current knowledge of the functions of various cell populations in hypersensitivity disorders, the book goes on (in Chapter IV) to discuss the molecular properties of allergens, and suggests that the dose, the antigenic valency and the physico-chemical state of the antigen determine the type of immune response induced. This is followed by a contribution on immunoglobilin E biosynthesis and the mechanisms of immunoglobulin E-mediated hypersensitivity, including the regulation of antibody formation by suppressor T lymphocytes. There is a most interesting chapter on the genetics of allergy. Allergic disorders exhibit a familial prevalence and a close association can be detected between HLA antigens and allergen responses in patient populations. The last part of the book is an extensive review of current concepts of the pathogenesis and management of clinical diseases with mechanisms based on atopy or hypersensitivity. The chapters on drug allergy and infiltrative pulmonary disease are most pertinent to those involved with pharmaceutical and allied products or concerned with occupational risks.

As a whole the volume provides both an excellent background for the understanding of allergic diseases and an extensive review of current knowledge of the mechanisms of various types of hypersensitivity induced by many different allergens.

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Information Section

ARTICLES OF GENERAL INTEREST

DIOXIN: CARCINOGENICITY

Carcinogenicity and reproductive effects are the two biological aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) that have, up to now, rather monopolized the column inches. In neither area is our knowledge at an advanced stage. We hope to cover dioxin's reproductive effects in a future article; in this issue we turn our attention to the animal carcinogenicity data. Earlier this year (*Cited in F.C.T.* 1980, **18,** 541, we reported a Swedish study suggesting that 2,4,5-T (contaminated with dioxin) and related herbicides may be carcinogenic in man. Although the study did not allow the contribution of dioxin to be determined, these results are clearly very worrying.

In 1977, the initial attempt of the International Agency for Research on Cancer to evaluate the carcinogenic potency of dioxin floundered on the paucity of long-term data (*IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man.* Vol. 15; IARC, Lyon, 1977, p. 41). At that time, an interim report of a long-term feeding study (Van Miller & Allen, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1977, **36,** 396) involving groups of ten male Sprague–Dawley rats indicated that dioxin was in fact carcinogenic. Of the 12 rats dead at wk 65, three given a diet containing 5 ppb dioxin ($b = 10^9$) had developed aplastic anaemia. Tumours found in six of the other nine rats that had died during this period after receiving diets containing 0·005, 0·05, 0·5, 1 or 5 ppb were identified as two carcinomas of the kidney, two carcinomas of the liver, one skin carcinoma and one angiosarcoma. According to an updated report, which included data through to the end of the 95-wk study (Van Miller *et al. Chemosphere* 1977, **9,** 537), no tumours had developed in the group given the lowest dose (fed a diet containing 0·001 ppb dioxin) or in the ten control rats. However neoplasms were seen in a total of 38% of the animals given subacute doses, that is in some rats all the dose levels from 0·005 ppb dioxin (equivalent to 0·15 ng/kg body weight/day) upwards. The tumours affected a diversity of tissues, including those of the lung, brain and testes in addition to those mentioned above. A long-term feeding study conducted at Dow Chemical research laboratories (Kociba *et al. Toxic. appl. Pharmac.* 1978, **46,** 279) has confirmed dioxin's carcinogenicity in Sprague–Dawley rats. Groups comprising 50 animals of each sex were maintained on diets containing 2·2, 0·21 and 0·022 ppb dioxin for 2 yr. At the highest dose level, equivalent to 0·1 μg/kg body weight/day, there was an increased incidence of hepatocellular carcinoma (in the females only) and of squamous cell carcinomas of the lung, hard palate/nasal turbinate and tongue, but a reduction in the incidence of a number of age-related lesions, including tumours of the pituitary, uterus, mammary gland, pancreas and adrenal gland. Although hepatocellular liver nodules, commonly classified as a precarcinogenic change, were seen in the animals receiving 0·21 ppb dioxin, there was no similar evidence of carcinogenicity at 0·022 ppb (1 ng/kg body weight/day).

The preliminary indications are that the mouse may also prove to be susceptible to dioxin's carcinogenic activity. In a Hungarian study (Tóth *et al. Nature, Lond.* 1979, **278,** 548), groups of 45 male mice (Swiss/H/Riop strain) were given dioxin at levels of 0·007, 0·7 and 7 μg/kg body weight by gavage once a week for 1 yr, and then kept for the rest of their lives untreated. Liver tumours occurred in 29, 48 and 30% of the low-, middle- and high-dose groups respectively and in 18% of the 38 controls. A statistically significant increase in liver-tumour incidence ($P < 1\%$) could only be demonstrated, therefore, in the group receiving 0·7 μg/kg. Since the toxicity associated with the highest dioxin treatment resulted in a considerable reduction in the average life-span of the animals (424 days), a simple statistical comparison with the control group, which had an average life-span of 588 days, was not appropriate. Lymphomas were seen in 27% of the mice given 0·7 μg/kg and in 23% of those receiving 0·007 μg/kg, compared with a control incidence of 16%.

Dioxin demonstrated only a weak initiating ability when tested by DiGiovanni *et al.* (*Bull. envir. Contam. Toxicol.* 1977, **18,** 552) in a two-stage skin carcinogenesis study. A single local application (2 μg/mouse, in acetone) to a group of 30 female Charles River CD-1 mice, followed after a gap of 1 wk by a 32-wk period of twice-weekly treatment with a stronger promoter, 12-*O*-tetradecanoylphorbol 13-acetate, produced papillomas in 14% of the 21 surviving mice (an average of 0·1 papillomas/mouse). Under similar experimental conditions, 7,12-dimethylbenz[*a*]anthracene (DMBA, 2·5 μg/mouse) induced an average of two papillomas/mouse, 40% of the 29 survivors being affected. When both DMBA and dioxin were applied simultaneously the effect was roughly additive. However, when dioxin (1 μg) was applied 3 days prior to initiation with DMBA, the activity of DMBA was decreased by 93% (Berry *et al. Ann. N.Y. Acad. Sci.* 1979, **320,** 405). The latter group also tested dioxin's promoting activity (Berry *et al. loc. cit.*) using mouse skin. Dioxin applied at 0·1 μg twice weekly for 30 wk did not act as a promoter, after an initiating dose of 200 nmol DMBA/mouse.

Carcinogenic in the rat, and possibly so in the mouse, is dioxin's score so far. The two rat studies, both using the Sprague–Dawley strain but one only reported in brief, have produced some comparable

results at the higher dose levels (2–5 ppb in the diet), in that lung and liver tumours were common findings, but not at lower doses, where Van Miller and his colleagues observed a diverse spectrum of tumours at dietary levels as low as 0·005 ppb, in contrast to the Dow scientists who were unable to detect a carcinogenic response at 0·022 ppb in spite of their much larger treatment groups. Long-term animal studies involving the rat and mouse have also been conducted at the US National Cancer Institute and the final report is being drafted. Nevertheless, on the evidence of a three-generation reproduction study demonstrating adverse effects in the F1 generation that were not present in the F0 generation (Murray *et al. Toxic. appl. Pharmac.* 1979, **50**, 241), a sounder estimate of carcinogenic potency may require a long-term study incorporating *in utero* exposure . The NCI experiments are unlikely to have used this type of protocol.

Again an understanding of mechanisms is called for. Ring epoxidation *in vivo* is a theoretical activation step but metabolism, if it does occur, is very slow. Dioxin is only selectively mutagenic in bacterial systems and is a weak inducer of chromosomal aberrations *in vivo*. From a structure–function point of view, the planar configuration of dioxin has invited comparison with the polynuclear aromatics or acridines. However, unlike the carbon atoms bridging the benzene rings in the tricyclic aromatic carcinogens, the oxygen atoms of the dioxin molecule do not form part of a conjugated bond system, and there is therefore no structural analogy on which to base an expectation of carcinogenicity (Hay, *Nature, Lond.* 1977, **269**, 468).

[J. Hopkins—BIBRA]

INHALATION STUDIES ON MMA

There has been some concern about the effects on operating-theatre staff of exposure to methylmethacrylate (MMA) which is used as a cement for fixing prosthetic devices. Furthermore, those involved in the manufacture and processing of MMA are also exposed to MMA vapour. The papers reviewed below deal with inhalation studies on MMA and several present sequels to earlier studies on which we have commented.

One such paper (McLaughlin *et al. Archs envir. Hlth* 1979, **34**, 336) reports a toxicity study in which mice inhaled high doses of MMA for a relatively short period. A few years ago, this group exposed pregnant mice to atmospheric concentrations of 1330 ppm MMA for 2 hr twice daily on days 6–15 of pregnancy (*Cited in F.C.T.* 1979, **17**, 421). They detected no effect on the foetus or on reproductive parameters, but the dams showed some signs of acute pulmonary inflammation, which was tentatively attributed to a secondary effect of the ether overdose used to kill them. The lack of any specific teratogenic finding has recently received support from a study in rats (Nicholas *et al. Toxic. appl. Pharmac.* 1979, **50**, 451) involving a very severe exposure—100 mg/litre air (24,440 ppm) for 17 or 54 min daily—on days 6–15 of pregnancy. These exposures were toxic to both dams and foetuses, as evidenced by an increase in early foetal deaths, reductions in foetal weight and length, and delayed ossification. However, signs of malformation effects were limited to some vertebral and rib anomalies in the group exposed for the longer periods, and it seemed likely, in view of the highly toxic exposures (each equivalent to 75% of the LT_{50}), that these were the result of a non-specific retardation of foetal maturation rather than directly induced malformations.

To check their assumption that the ether overdose was implicated in the development of pulmonary inflammation in their MMA-exposed mice, McLaughlin *et al.* (*loc. cit.*) exposed non-pregnant mature female mice to an average concentration of 1520 ppm MMA, again for 2 hr twice daily, and at the end of the 10-day exposure period killed them by cranial concussion or sudden cervical spine fracture. Like the heart, liver and kidney tissues, the lungs of the treated mice did not differ histologically from those of the controls; pulmonary tissue of both groups showed only occasional non-specific focal cellular reactions. The authors point out that this negative result was obtained with concentrations over five times as high as those found in operating theatres during total hip replacements and the treatment period was much longer than the exposure times encountered during such surgery.

Another investigation on which data have just been published was concerned not with the very specialized type of exposure likely to be encountered during the use of MMA in surgical procedures, but with the levels of exposure encountered industrially during MMA manufacture and processing and subject to a current TLV in the UK and USA of 100 ppm (410 mg/m^3). The first paper relating to this series of inhalation studies was published in 1976 and reported the effects of exposing rats to an atmospheric level of 116 ppm MMA for 8 hr/day on 5 days/wk for 3 or 6 months (*Cited in F.C.T.* 1977, **15**, 257). These effects included a reduction in intestinal transit rates, attributable to an inhibition of spontaneous motor activity in the small intestine, and a marked reduction in body weight and in abdominal fat in rats exposed for 3 months. Body weights and fat deposits were still reduced after the 6-month exposure but the effect was less severe. Interference with intestinal transit was seen also in dogs exposed briefly to a high concentration (2000 ppm) of MMA (Tansy *et al. J. pharm. Sci.* 1977, **66**, 613).

Further rat studies were undertaken (*idem, Envir. Res.* 1980, **21**, 108) to investigate the mechanism underlying the reductions in weight and body fat, the three possible causes having been identified as changes in feeding behaviour, impairment of the absorptive-motor functions of the gastro-intestinal tract and alterations in fat metabolism. Body weights, food and water intakes and excretion rates were determined in mature males exposed for 3 months to 116 ppm MMA for 7 hr/day on 5 days/wk and, like the controls, deprived of food during the exposure periods. Terminal blood-serum analyses showed a significant decrease in total bilirubin and an increase in total cholesterol—two findings of which the earlier study had given only a slight indication. In contrast, there was no visual evidence of any reduction in vis-

ceral or subcutaneous fat in these treated animals nor any significant difference in body weights or in weekly food and water intakes between this group and the controls. While faecal excretion was similar in the two groups at weekends, when no exposure occurred, it was very variable on the days on which the rats were treated with MMA, and the mean weekly values expressed as a percentage of food intake were higher for the treated group than for the controls.

The authors attributed the lack of agreement between the body-weight and fat-deposit findings in the two studies to the use of older animals and an *ad lib.* feeding regime in the second test. In the first study, diet was restricted, although from months 3 to 6 more food was available per rat because of the removal of the group given the 3-month exposure. Relevant to this demonstration of the combined effect of MMA and dietary restriction on rats at a stage of rapid growth is the authors' finding that MMA-exposed rats tend to have higher oxygen-consumption values; work is in hand to determine whether MMA exposure has a direct effect on fat metabolism when the food intake is restricted. Although the faecal excretion data indicate that the effects of MMA exposure on intestinal motility are only transitory, the persistent repetition of this situation may also be of significance when superimposed on the other factors mentioned above.

No tumours were found in any of the rats exposed to 116 ppm MMA for 3 or 6 months and no untoward effects were detected during routine histology of the heart, kidney, spleen, stomach, small intestine and adrenals (*idem, ibid* 1980, **21,** 117). Occasional slight lung damage was seen but this occurred similarly in the control group. However, after 6-months all of the MMA-exposed rats, but none of the controls, had small areas of focal haemorrhage in the tracheal mucosa, associated with an absence of cilia and microvilli from the epithelial cells. Similar ciliary denudation was demonstrated in frogs exposed to 400 ppm MMA, although not to 116 ppm, and was shown in that species to be associated with a significant impairment of propulsive function. In rats exposed to 1000 ppm MMA for a total of 56 hr over a 7-day period (an exposure nearer to that used by McLaughlin *et al. loc. cit.*) there was obvious lung damage, which included pleural adhesions and some fibrosis, oedema and indications of emphysema. Such changes again tended to occur also in controls, but they were then less severe. Some inconsistent and equivocal suggestions of liver damage were detected in the rats exposed to 116 ppm MMA and also in mice exposed to 100 or 400 ppm. In these mice, a significant reduction in pentobarbitone induction and sleeping times after total MMA exposures of 160 hr indicated some alteration in liver function.

These additional studies have tied up some of the loose ends in the earlier work, but we have underlined some further questions that remain unanswered. The current TLV for MMA was set with the main aim of preventing discomfort from irritation, since much higher levels were thought to be required to produce systemic effects. However, the reported results of exposing rats to atmospheric concentrations close to the TLV suggest some possibility of chronic effects from such concentrations, although further investigation of the cellular responses will be required before conclusions can be drawn. Then, well-conducted epidemiology studies will still be required to establish the relevance of these animal studies to man.

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ABSTRACTS AND COMMENTS

NATURAL PRODUCTS

Role of aflatoxins in Reye's syndrome

Ryan, N. J., Hogan, G. R., Hayes, A. W., Unger, P. D. & Siraj, M. Y. (1979). Aflatoxin B_1: its role in the etiology of Reye's syndrome. *Pediatrics, Springfield* **64,** 71.

There has been considerable interest in the role of aflatoxins in the pathogenesis of Reye's syndrome, an acute and often fatal disease that is characterized by a combination of acute encephalopathy with fatty degeneration of the viscera, including the liver (*Cited in F.C.T.* 1979, **17,** 409). The group cited above has previously reported that the blood of two children with Reye's syndrome contained aflatoxin B_1 during the acute stage of the disease (Hogan *et al. Lancet* 1978, **I,** 561). The present report discusses the association between aflatoxins and Reye's syndrome in relation to these and six other cases.

The eight patients were admitted to a US paediatric neurology clinic between January 1975 and May 1978. Blood samples were taken for aflatoxin analysis from all the children (five) with clinically suspected Reye's syndrome who were admitted between January 1976 and May 1978. Aflatoxin B_1 was present in the blood of two of the children at levels of 31·3 and 11·93 ng/ml. However no aflatoxin was detected in blood taken from 37 controls. These controls included children of matched age, socio-economic status and geographic location who were admitted to the clinic with coma or who were treated in the outpatients department. Only one patient in the control group had liver disease (glycogen-storage disease).

All of the children with Reye's syndrome died and an autopsy was carried out on seven of them. Aflatoxin B_1 was detected in the livers of six of the children, at levels between 2·23 and 17·33 ng/g; the seventh liver sample was inadequate for a determination. Liver samples from twelve children who had died of causes other than Reye's syndrome, including two with known liver disease, were also analysed. Aflatoxin was detected in the liver of only one of these controls—an infant with hydrocephalus and with a ventriculoperitoneal shunt who had had bacterial meningitis.

The source of aflatoxin in the children with Reye's syndrome was not known. Presumably the older children might have ingested aflatoxin-contaminated nut or cereal products. However, five of the subjects were infants less than 8 months old. According to the authors, infant cereal foods and hypoallergenic milk substitutes may be sources of aflatoxins, and one of the infants was being fed the latter. The authors cite a report that, in the USA, children with Reye's syndrome come primarily from lower-socio-economic urban environments (Huttenlocher & Trauner, *Pediatrics, Springfield* 1978, **62,** 84) and point out that under such conditions children are likely to be more susceptible to enteroviruses, to come into contact with aflatoxin-contaminated food and to be fed cereals and adult foods at an earlier age. If this is so, why was no aflatoxin detected in the control children who had been matched for age and socio-economic status? Aflatoxin is rapidly removed from the blood and is excreted, mainly in the bile. Unger *et al.* (*Toxic. appl. Pharmac.* 1977, **41,** 523) reported that cholestasis occurred in rat liver that had been perfused with aflatoxin B_1 for 120 min, thus reducing the biliary excretion of aflatoxin B_1. Light microscopy revealed no pathological changes in the liver. If a similar situation exists in humans, aflatoxin would not be detectable in the blood unless significant liver damage had already occurred. If it is assumed that a small portion of ingested aflatoxin remains in the liver cells, then repeated ingestion of aflatoxin B_1 could result in the accumulation of cytotoxic levels. This inapparent liver injury could be potentiated by a viral infection with resultant destruction of the liver cells. The authors consider that the presence of aflatoxin in the blood and liver of patients with Reye's syndrome represents just one of several interrelated factors that are responsible for the development of the disease.

AGRICULTURAL CHEMICALS

Spermatogenesis upset by 1,2-dibromoethane

ElJack, A. H. & Hrudka, F. (1979). Pattern and dynamics of teratospermia induced in rams by parenteral treatment with ethylene dibromide. *J. Ultrastruct. Res.* **67,** 124.

Hrudka, F. & ElJack, A. H. (1979). The effect of ethylene dibromide on differentiation of the acrosome, nucleus, and transient nuclear appendages in ram spermatids. *J. Ultrastruct. Res.* **67,** 135

1,2-Dibromoethane (DBE; ethylene dibromide) has been used as a fumigant for soil, and for grain and some other food crops. However, several studies have indicated that it may have adverse effects on those exposed to it. Rats and mice given DBE in corn oil by gavage developed squamous-cell carcinomas and other tumours (*Federal Register* 1978, **43,** 52775). Furthermore DBE produces sperm defects in the bull (*Cited in F.C.T.* 1965, **3,** 864; Amir & Volvani, *Fert. Steril.* 1967, **18,** 144), and in chickens significantly reduces egg size and egg fertility, although it has no

effect on sperm characteristics or on fertility in mature cocks (Alumot *et al. Poult. Sci.* 1968, **47**, 1979). Teratospermia in rams has now been investigated in the studies cited above.

In the first investigation cited, sc injections of 7·8, 9·6 or 13·5 mg DBE/kg body weight/day administered to rams over 12 days induced transient teratospermia after a delay of 2–3 wk. Sperm motility declined 5 wk after treatment began, reached its lowest point in wk 9–10 and returned to pretreatment values after about 15 wk. Nitroblue tetrazolium reductase activity was determined in two of the rams and showed the same trend in both; it began to decline in wk 5 and reached its lowest level between wk 9 and 10. Acrosomal abnormalities first appeared in the granular or vesicular structure of the principal segment of the organelle during wk 5 after the beginning of DBE treatment. Gradually abnormalities began to appear in the apical segment. The segment enlarged and its protruding apical tip folded back over the dorsal or ventral aspect of the spermatozoon head. The plasma membrane disappeared and the duplicated acrosomic membrane broke into minute vesicles. Nuclear abnormalities included rolled, pyriform, round, macrocephalic, microcephalic and twin heads. Nuclear crests occurred frequently. Severely deformed nuclei showed uneven chromatin packing, irregular cavities and granular nucleoplasm. The highest incidence of nuclear changes occurred 12–14 days after the maximum incidence of acrosomal defects. Of the sperm abnormalities observed, defects of the mitochondrial sheaths were the least common. The mitochondrial sheath defects included aplasia, segmental hypoplasia and deranged organization.

The second paper cited above describes the ultrastructural changes that were observed in the spermatids of rams treated sc for 12 days with 13·5 mg DBE/kg/day. Orchidectomy was carried out on day 22, 27, 32 or 38 after the beginning of treatment. Acrosomal damage appeared during the cap phase of spermatogenesis and included aplasia, hypoplasia or hyperplasia, cap deformation, vesicular inclusions in the acrosomic matrix and lesions in the acrosome attachment to the nucleus. Some of these changes could be related to changes in the morphology and activity of the Golgi complex. Abnormalities in the differentiation of the nuclei occurred during the third phase of spermatogenesis and included abnormal chromatin condensation, disturbed elimination of nuclear sap and aberrations in the shapes of the nuclei. Nuclear metamorphosis appears to depend on the acrosome, which is the prerequisite for cell polarization and which in turn is essential for the differentiation of the caudal manchette and the Sertoli cell mantle. DBE induces abnormal spermatid development specifically by affecting the differentiation of the acrosome and nucleus. The authors believe that the acrosomic defects are the result of an aberrant activity of the Golgi complex affected by DBE earlier in spermatogenesis.

FEED ADDITIVES

Transplacental effect of DES in rats

Napalkov, N. P. & Anisimov, V. N. (1979). Transplacental effect of diethylstilbestrol in female rats. *Cancer Lett.* **6**, 107.

Vorherr, H., Messer, R. H., Vorherr, U. T., Jordan, S. W. & Kornfeld, M. (1979). Teratogenesis and carcinogenesis in rat offspring after transplacental and transmammary exposure to diethylstilbestrol. *Biochem. Pharmac.* **28**, 1865.

Vaginal adenocarcinoma has been found to occur in young girls exposed prenatally to diethylstilboestrol (DES) and studies have shown that exposure to DES *in utero* induces vaginal abnormalities (*Cited in F.C.T.* 1975, **13**, 589). Further animal studies of the transplacental effect of DES are therefore important.

In the first study cited above, 11 rats were given single sc injections of 1 mg DES/kg body weight on day 19 of gestation. The female offspring were taken from their mothers 3 wk after birth and were studied throughout their lives. The offspring of 17 untreated rats were observed as controls. In order to study the effects of DES on hypothalamic–pituitary–ovarian function, at the age of 3–4 months some of the offspring were hemicastrated, after which they were given sc injections of 0·57 μg DES/day for 7 consecutive days and were killed on day 8. Vaginal smears indicated persistent oestrus in the adult rats exposed to one dose of DES prenatally. In rats untreated during pregnancy, DES suppressed the compensatory ovarian hypertrophy that was induced by hemicastration. However, DES administered after hemicastration had no effect on compensatory ovarian hypertrophy in the rats exposed to DES *in utero*. Glucose tolerance was decreased in the prenatally-exposed offspring. Tumours developed in 14 out of 18 (78%) of the offspring of DES-treated rats, but in only nine out of 34 (26%) of the control rats. Tumours of the ovaries and endometrium occurred only among the DES-exposed rats. No cervical or vaginal tumours were found in the treated or control rats. It is suggested that exposure to DES might impair the sex differentiation of the hypothalamus in female rats and that the resulting hormonal or metabolic shifts might promote carcinogenesis.

In the second study cited, rats were given sc injections of 0·015–0·6 mg DES/kg body weight on days 13, 16, 18 and 20 of gestation in order to determine the effects of DES on urinogenital development. Three lactating rats, including two given DES during gestation, were given doses of 0·2–0·8 mg DES/kg body weight sc on intermittent days during the 3 wk following delivery. Another lactating rat was given 10 mg DES/kg/day on days 9 and 20 *post partum*. Five of the DES-treated rats delivered 31 live offspring, 20 of which survived. Nineteen of these were observed throughout their lifespan. Three of them had

been exposed to DES *in utero*, six were exposed *via* the mothers' milk and ten were exposed both pre- and postnatally. DES exposure during gestation and/or during the first 4 days of lactation disturbed the development and differentiation of the female urinogenital sinus epithelium, while in the males its major effect was the inhibition of the growth and descent of the testes and of the development of the Wolffian structures. No malignancy was observed in DES-exposed male offspring, but malignant tumours, including vaginal cell carcinoma and endometrial and ovarian adenosarcomas, occurred in four out of ten of the female offspring. No genital malignancy was observed among the controls.

ENVIRONMENTAL CONTAMINANTS

A brush with mercury

Kirschbaum, B. B. & Oken, D. E. (1979). The effect of mercuric chloride on renal brush border membrane. *Expl mol. Path.* **31**, 101.

In the last issue (*Cited in F.C.T.* 1980, **18**, 552), we reported the results of an ultrastructural study of nephritis induced in Brown Norway rats by mercuric chloride. Several workers have shown that acute renal failure induced by mercuric chloride is accompanied by tubular transport abnormalities and that cellular injury is most marked in the proximal tubule (*ibid* 1975, **13**, 468). Several renal enzymes are affected by mercuric chloride (Price & Kempson, *Biochem. Soc. Trans.* 1975, **3**, 294) and brush-border enzyme disturbances have been noted in earlier studies (Zalme *et al. Virchows Arch. Abt. B Zellpath.* 1976, **22**, 197).

In an attempt to examine further the effects of mercuric chloride on brush-border enzymes, female Sprague–Dawley rats were given a single sc injection of 4·7 mg mercuric chloride/kg body weight. Microvilli were isolated from kidney samples taken at intervals and five brush-border enzymes were assayed. By days 3–5 after injection, alanine aminopeptidase, γ-glutamyl transpeptidase and alkaline phosphatase levels fell to 50% or less compared with the saline-treated controls. The levels remained depressed at day 10 despite almost complete restoration of glomerular filtration by day 5. Maltase activity initially increased but then gradually declined to control values by day 4 after dosing. ATPase activity showed a progressive rise to levels 3·1 times greater than in the controls by day 10. In addition, electrophoresis of sodium dodecylsulphate-solubilized brush-border membranes revealed distinct differences in protein distribution between treated and control rats.

These results differ quite considerably from those of Price & Kempson (*loc. cit.*) or Zalme *et al.* (*loc. cit.*) and it is thought that this might reflect the different methods used in the enzyme assays. However, all the studies indicate that mercuric chloride induces significant membrane damage and this is reflected in the non-uniform disturbances in brush-border enzyme activity. The functional significance of these changes is not clear, especially in view of the observation that the recovery of renal function precedes the return of the brush-border enzyme activity to normal levels.

A whiff of nitrogen dioxide...

Kerr, H. D., Kulle, T. J., McIlhany, M. L. & Swidersky, P. (1979). Effects of nitrogen dioxide on pulmonary function in human subjects: an environmental chamber study. *Envir. Res.* **19**, 392.

The widespread atmospheric pollutant nitrogen dioxide (NO_2) has been shown to induce pulmonary damage in rats (*Cited in F.C.T.* 1970, **8**, 219) and dogs (*ibid* 1972, **10**, 727) at exposure levels of only a few ppm. In the study cited above the effects of exposing humans to low levels of NO_2 were investigated.

Thirteen subjects with asthma, seven with chronic bronchitis and ten normal healthy subjects were studied. The tests were carried out in a controlled-environment chamber. On day 1 the subjects breathed filtered clean air for 2 hr and on day 2 they breathed air containing NO_2 for 2 hr at the same time of day. For 15 min during the first hour of the tests they performed light exercise using a bicycle ergometer. The NO_2 levels were monitored throughout each subject's exposure period and the mean levels of NO_2 were found to range between 0·49 and 0·51 ppm. Pulmonary-function tests (spirometry, plethysmography and the single-breath nitrogen elimination rate) were carried out before and after exposure. Tests for pulmonary resistance and compliance were carried out only at the end of the tests.

Seven of the asthmatic subjects experienced mild chest tightness, burning of the eyes, headache or dyspnoea. One subject with bronchitis and one normal subject developed slight rhinorrhoea. A significant decrease in quasistatic pulmonary compliance was observed on the second test day in the normal volunteers, but no significant decrease in dynamic pulmonary compliance was observed for any of the three groups. No significant changes in pulmonary-function tests were observed in the asthmatic or bronchitic groups when they were considered separately. However when considered together there were significant changes in quasistatic pulmonary compliance, distribution of ventilation and static lung volumes. These changes suggested a change in the elastic properties of the lungs. However since some of the changes were observed before exposure began they could not be attributed to NO_2. It is therefore concluded that no significant alteration in pulmonary function is likely to result from exposure to 0·5 ppm NO_2 over 2 hr in healthy, asthmatic or bronchitic subjects. Although a double-blind study of the effects of such low levels of NO_2 would be desirable it would not be practical since the odour of NO_2 is detectable at 0·5 ppm.

...A puff of sulphur dioxide...

Murray, F. J., Schwetz, B. A., Crawford, A. A., Henck, J. W., Quast, J. F. & Staples, R. E. (1979). Embryotoxicity of inhaled sulphur dioxide and carbon monoxide in mice and rabbits. *J. envir. Sci. Hlth* **C13**, 233.

Murray, F. J., Schwetz, B. A., Nitschke, K. D., Crawford, A. A., Quast, J. F. & Staples, R. E. (1979). Embryotoxicity of inhaled sulphuric acid aerosol in mice and rabbits. *J. envir. Sci. Hlth* **C13**, 251.

Sulphur dioxide (SO_2) is a common atmospheric pollutant, which arises from the combustion of sulphur-bearing coals and petroleum fuels. Studies of effects have concentrated mainly on its effects on the respiratory tract and on pulmonary function (*Cited in F.C.T.* 1973, **11**, 1136). However the two papers cited above describe investigations of the teratogenic potential of SO_2 and of another common air pollutant, sulphuric acid aerosol (H_2SO_4), which is formed by the oxidation of SO_2 under certain atmospheric conditions.

In the first study, groups of 32 and 35 male CF-1 mice were exposed for 7 hr/day to 25 ppm SO_2 or to 25 ppm SO_2 plus 250 ppm carbon monoxide (CO), respectively, on days 6–15 of gestation. Groups of 20 New Zealand White rabbits were exposed to 70 ppm SO_2 or to 70 ppm SO_2 plus 250 ppm CO for 7 hr/day on days 6–18 of gestation. In the second study, groups of 35 CF-1 mice and of 20 New Zealand White rabbits were each exposed to 5 or 20 mg H_2SO_4/m^3 for 7 hr/day on gestation days 6–15 (mice) or 6–18 (rabbits). All of the experiments were carried out in inhalation chambers, with an air flow of 800 litres/min, and groups of control animals were exposed to filtered room air. In both studies the mice and rabbits were killed on day 18 and 29 of gestation, respectively.

There were signs of slight maternal toxicity in both mice and rabbits exposed to SO_2, to SO_2 plus CO and to the higher level of H_2SO_4. Food consumption was reduced in all these groups, and exposure to 20 mg H_2SO_4/m^3 resulted in a significant reduction in liver weight (absolute and relative) in mice and in maternal weight gain during the first few days of exposure in rabbits. The mean carboxyhaemoglobin levels in both mice and rabbits on days 1 and 3 of treatment were 1–2% and 15–17% with exposure to SO_2 and to SO_2 plus CO, respectively. Histological examination of the nasal turbinates and tracheae of rabbits exposed to H_2SO_4 indicated a dose-related increase in the incidence of subacute rhinitis and tracheitis. The incidence of pregnancy was not significantly affected by any of the treatments, except for an increase in the proportion of early resorptions (detected by sodium sulphide stain) in the mice exposed to SO_2 plus CO.

The foetuses of mice exposed to SO_2 or to SO_2 plus CO weighed significantly less than the controls and the foetal crown–rump length was also less in the group treated with SO_2 plus CO. However the treatments did not affect foetal weight or measurements in the rabbits, although the rabbits exposed to the combination of gases had significantly more late resorptions. In both species, no one type of malformation occurred at a greater incidence among foetuses in the treated groups than in the controls, but the incidence of major malformations, when considered collectively was slightly increased ($P = 0.14$) among the foetuses of mice inhaling SO_2 plus CO. Minor skeletal variations occurred more frequently among the foetuses of both mice and rabbits treated with SO_2 or SO_2 plus CO. Treatment with either level of H_2SO_4 had no effect on the mean numbers of foetuses/litter or resorptions/litter, nor on the mean foetal weights or lengths in mice or rabbits. Neither was there any increase in malformations among offspring of the treated animals, apart from an increase in the incidence of small non-ossified areas in the skull bones of the foetuses of rabbits that inhaled 20 mg H_2SO_4/m^3. It is therefore concluded that at the levels used in these experiments, the inhalation of SO_2, of SO_2 plus CO or of H_2SO_4 does not have teratogenic effects in mice or rabbits.

... And a lungful of sulphates

Avol, E. L., Jones, M. P., Bailey, R. M., Chang, N. N., Kleinman, M. T., Linn, W. S., Bell, K. A. & Hackney, J. D. (1979). Controlled exposures of human volunteers to sulfate aerosols. Health effects and aerosol characterization. *Am. Rev. resp. Dis.* **120**, 319.

Sulphur dioxide and other sulphur compounds are converted to sulphates in the atmosphere. However, knowledge of the effects of ambient levels of sulphates on human health is limited. In a 1978 review, Ferris (*J. Air Pollut. Control Ass.* 1978, **28**, 482) concluded that the data on the health effects of sulphates were insufficient to set an atmospheric standard for these compounds. In the study cited above, volunteer human subjects were exposed to a series of sulphate aerosols at concentrations equivalent to the highest that have been encountered in the Los Angeles atmosphere.

The tests were carried out on normal and asthmatic subjects. In the tests on ammonium sulphate, 'sensitive' subjects were also studied; these were volunteers who had previously shown unusually definite reactions in controlled exposures to ozone. Since there was no difference in the response to ammonium sulphate between the 'sensitive' and the normal volunteers the two groups were combined in subsequent exposures. The experiments were carried out in a stainless-steel chamber, continuously supplied with 14 m^3 purified air/min. The exposure concentrations and particle distributions of ammonium sulphate, ammonium bisulphate and sulphuric acid were based on the highest reported filter sample of particulate sulphate in the Los Angeles basin. Aerosol concentrations and size distributions in the chamber were monitored using an electrical aerosol analyser and an optical particle counter, both linked to a computer. Gravimetric and chemical analyses were carried out on filter samples collected during the experiments and analysed immediately after them. The subjects were given 1 or 2 days control exposure and then 2 or 3 consecutive days of sulphate-aerosol exposure. The exposure period was 2 hr and for the first 15 min of each 0·5 hr the volunteers took exercise at a level intended to double (approximately) their minute volume of ventilation. The subjects carried out lung function tests and completed symptoms questionnaires on entering the chamber and before they left it.

Exposure to ammonium sulphate (104–138 $\mu g/m^3$) at 85 or 40% relative humidity produced no obvious changes in pulmonary function in five normal subjects (mean age, 37·2 $\pm$ 10 yr) although there were signs of heat stress at the higher humidity. The group of five

sensitive volunteers (mean age 39·8 ± 11 yr) showed small but significant variations in pulmonary function at both high and low humidity but these did not seem to be related to exposure to ammonium sulphate (109–182 $\mu g/m^3$). No adverse effects of ammonium sulphate aerosols (325–337 $\mu g/m^3$) were observed in six asthmatic subjects (mean age, 48·7 ± 13 yr) who were exposed, as were all the subjects tested with the other sulphate aerosols, only at the lower humidity. Seven normal subjects (mean age, 33·1 ± 10 yr) were exposed to ammonium bisulphate at total-suspended-particle (TSP) levels of 129 $\mu g/m^3$ (concn 47 $\mu g/m^3$) on day 1 and of 139 $\mu g/m^3$ (concn 17 $\mu g/m^3$) on day 2. Six asthmatic subjects were exposed at TSP levels of 123 $\mu g/m^3$ (concn 46 $\mu g/m^3$) on day 1 and 138 $\mu g/m^3$ (concn 4 $\mu g/m^3$) on day 2. During the experiments it became apparent that a substantial portion of the aerosol was neutralized by subject-exhaled and chamber-background ammonia. No treatment-related changes in lung function were observed in either group. Exposure to sulphuric acid (TSP 234 $\mu g/m^3$, concn 101 $\mu g/m^3$ on day 1; TSP 264 $\mu g/m^3$, concn 111 $\mu g/m^3$ on day 2) had no effect on six normal subjects (mean age, 35·5 ± 10 yr). The six asthmatic subjects (mean age, 35·5 ± 14 yr) exposed to sulphuric acid at TSP levels of 248 $\mu g/m^3$ (concn 101 $\mu g/m^3$) and 233 $\mu g/m^3$ (concn 93 $\mu g/m^3$) on days 1 and 2, respectively, showed no lung-function changes that were significant for the whole group. However, two of the asthmatic volunteers showed possibly significant changes in respiratory resistance; their measured respiratory resistance did not change substantially in the control studies, but increased significantly during the period of exposure to sulphuric acid.

In this study few, if any, deleterious changes in pulmonary function resulted from exposure to ammonium sulphate, ammonium bisulphate or sulphuric acid. It seems therefore that sulphate aerosols at 'worst case' ambient levels would elicit at most only a minimal short-term adverse response. It is possible that the adverse health effects that have been attributed to sulphates in some epidemiological studies were in fact due to other coexisting pollutants.

Rhesus monkeys floored by PCB

Altman, N. H., New, A. E., McConnell, E. E. & Ferrell, T. L. (1979). A spontaneous outbreak of polychlorinated biphenyl (PCB) toxicity in rhesus monkeys (*Macaca mulatta*): clinical observations. *Lab. Anim. Sci.* **29,** 661.

McConnell, E. E., Haas, J. R., Altman, N. & Moore, J. A. (1979). A spontaneous outbreak of polychlorinated biphenyl (PCB) toxicity in rhesus monkeys (*Macaca mulatta*): toxicopathology. *Lab. Anim. Sci.* **29,** 666.

The migration of polychlorinated biphenyls (PCBs) from a floor sealant into feed was believed to be responsible for a spontaneous outbreak of 'wasting disease' in a group of laboratory rhesus monkeys. Sealant stored in a barrel since the construction of the animal unit (8 yr earlier) was found to contain less than 15 ppm PCBs and was non-toxic to three rats when fed at a level of 1 ml/wk for 8 months, while levels of more than 5000 ppm were found in sealant taken from the cage floor in some feed areas. It could not be established whether the stored sealant had actually been used in the animal house and it was thought most probable that the floor had become contaminated by contact with PCB-containing feed during an earlier experiment.

The clinical characteristics of the affected monkeys are described in the first paper cited above. The animals became very irritable and apprehensive. Photophobia developed, accompanied by palpebral oedema and crusty exudations around the eyelids. The oedema spread to the face, on which acne also developed. Other signs of toxicity included weight loss, alopecia, pruritus, gingivitis, fibrous thickening of the mandible, diarrhoea and trauma. There was a high incidence of abortions and stillbirths. Live offspring were small and weak and there was a high incidence of neonatal mortality. The surviving infants developed clinical signs similar to those of the adults at 10–14 days of age.

Seven monkeys were taken for detailed pathological examination. Clinically, four of these showed advanced signs of disease, one had mild to moderate signs, and two had minimal signs. Histopathological examination showed prominent thickening and cell-type changes in the Meibomian glands, hyperkeratotic folliculitis of the eyelash and hair follicles (particularly in the palpebral skin and the numerous regions of alopecia), and hyperkeratosis of the nail bed with associated onychogryphosis. Cystic dilation of the mucous glands of the tongue and squamous metaplasia of the associated ducts were present in some monkeys, and oesophagostomiasis was common. The stomachs of three monkeys showed thickening of the mucosa, with massive hyperplasia of the mucous epithelial cells and focal downgrowth into the submucosa very like that previously reported to result from the ingestion of low levels of PCBs (*Cited in F.C.T.* 1980, **18,** 553). Lesions were not present in the small intestine, but focal ulceration with haemorrhage and adjacent thickening were observed in the colon of two of these monkeys.

No hepatic abnormalities were seen but thickening and enlargement of the gall bladder and bile ducts, caused by hyperplasia of the epithelia and pseudocyst formation, and focal squamous metaplasia of the mammary glands each occurred in two animals. The appendicular muscles had become pale and atrophied. Decreased total serum protein levels and an increased albumin to globulin ratio were recorded in the monkeys that had been severely ill and a clear serosanguinous ascitic fluid was obtained from two of these animals. Mild to moderate anaemia occurred in both the clinically ill and normal monkeys and marked hypoplasticity of the bone marrow was observed in the former. No PCB-related lesions were found in the respiratory tract, genito-urinary tract, lymphoreticular system or central nervous system.

The epithelial changes observed closely resembled the symptoms of vitamin A deficiency in rhesus monkeys. Indeed, serum vitamin A levels varied between 20 and 200 $\mu g/dl$ and the mean tended to be lower than accepted normal values although the difference was not significant. Hepatic levels were reduced to one third of controls. The presence of low hepatic

levels of vitamin A indicates that a deficiency of this essential nutrient may be involved in part of the pathogenesis of the lesions. However such a deficiency must have arisen from impaired vitamin A metabolism, not from a dietary shortage. It is concluded that although vitamin A may have an important role in the pathogenesis of the lesions and in fertility problems, it may bear little relationship to the actual cause of death.

The renal carcinogenic and nephrotoxic effects of TRIS

Reznik, G., Ward, J. M., Hardisty, J. F. & Russfield, A. (1979). Renal carcinogenic and nephrotoxic effects of the flame retardant tris (2,3-dibromopropyl) phosphate in F344 rats and (C57BL/6N × C3H/HeN)F_1 mice. *J. natn. Cancer Inst.* **63**, 205.

This paper describes the renal pathology of the animals used in the NCI bioassay that established tris-(2,3-dibromopropyl) phosphate (TRIS) as an animal carcinogen and that led to a ban on its use as a flame retardant in children's clothing in the USA (*Federal Register* 1977, **42**, 18850). The dietary concentrations used in the bioassay were 100 or 50 ppm for groups of 55 male and 55 female rats and 1000 or 500 ppm for groups of 50 male and 50 female mice. For both rodent types, 55 animals of each sex were used as controls. Between 65 and 80% of the rats and mice survived to the end of the study (*c.* 104 wk).

Various non-neoplastic lesions were frequently observed in the renal tissues of both species and included foci of dilated and hyperplastic or dysplastic proximal convoluted tubules, often in the same areas as tumours. The dysplastic lesions appeared to be dose related in the females. The cells constituting these lesions often had a brush border and their cytoplasm filled the lumen of the tubule. Enlargement of the nuclei (up to ten times their normal size) and of the nucleoli and dispersion of the chromatin occurred in some proximal-tubular epithelial cells. In other tubules similar enlarged disorientated nuclei protruded into the lumen and their cells lacked brush borders and cytoplasm at the luminal margin. Other areas showed only thickening of the basement membrane and vacuolization of the cell cytoplasm.

In the rats, adenomas were observed in the renal tubular tissue of 30 out of 54 males and of four out of 54 females given the lower dose of TRIS, and in that of 27 and of 54 males and of 13 out of 54 females given the higher dose. In the mice, renal tubular adenomas were observed in five out of 50 males and three out of 50 females, and in 12 out of 49 and three out of 46 females in the low- and high-dose groups, respectively. Frequently multifocal in one kidney but seldom bilateral, these tumours appeared to originate from the proximal convoluted tubule, and consisted of cuboidal to columnar epithelial cells with round nuclei in a pale, sometimes granular, cytoplasm. Some cells were vacuolated with an eccentric nucleus. Within the tumour tissue, denuded or necrotic epithelial cells were often found in the lumen of the tubule and brush borders were absent. The basement membranes around tumours or of adjacent tubules were thickened and frequently interrupted.

Three male rats in the high-dose group developed tubular-cell carcinomas (adenocarcinomas) and six mice (one in the low-dose group and five in the high-dose group) developed the same lesion. The adenocarcinomas, which sometimes replaced almost all of the normal renal tissue, invaded the kidney capsule and, in contrast to the adenomas, were not clearly demarcated from the remaining kidney cortex. The growth patterns were papillary, tubular or compact in nature. In the mice these neoplasms often contained single or multiple cyst-like structures filled with a fine flocculent material. The cells surrounding these cysts had eccentric, and often enlarged, nuclei with pronounced vacuolization of the cytoplasm. Some of the tumours had pronounced haemorrhages that formed erythrocyte-filled cavities into which papillary extensions of the marginal tissues protruded. Compared with the adenomas, the ratio of nuclear to cytoplasmic material was increased in the adenocarcinomas of both species.

TEST PROCEDURES

Inside teratogenicity

Snow, M. H. L. & Tam, P. P. L. (1979). Is compensatory growth a complicating factor in mouse teratology? *Nature, Lond.* **279**, 555.

Teratogenicity studies frequently involve the administration of the test compound to pregnant females by one of several routes followed by autopsy at about 24 hr before parturition. Although such methods are efficient, they neither determine the immediate response of the embryo to the substance nor the capacity of the embryo to regulate or recover from the damage. Thus developmental disturbances that might be manifest only postnatally could go unnoticed. The authors cited above have investigated this possibility.

The antibiotic mitomycin C (MMC) is teratogenic at high doses in rodents, producing exencephaly, spina bifida and skeletal and other non-nervous-system defects in some strains of mice. Pregnant random-bred Q-strain mice were given a single ip injection of 100 or 150 μg MMC/mouse on day 6·5 or 7 *post-coitum*. Data for the two dose levels were pooled since there was no noticeable difference in response. From 1·5 to 6 hr after the injection dead cells were observed in the embryos and mitotic activity decreased. Embryos examined at day 7·5 were morphologically normal but very small. Their cell numbers were found to be reduced to about 14% of the normal level but mitotic activity had increased again and was higher than normal. This high mitotic activity seemed to reflect accelerated compensatory growth. At day 8·5 the embryos were still small and were morphologically retarded. The mitotic index was high throughout

the embryo. It seemed clear to the authors that the embryos would die, but by day 10·5 although still small they were once again morphologically normal. By day 13·5 the only remaining evidence of damage was a significantly low mean foetal weight.

At parturition the litters of treated mice were slightly decreased in size. Newborn mice were not weighed but the treated mice were not noticeably smaller than the controls. Microphthalmia was seen in 26% of the 14-day-old mice but there were no other abnormalities. Growth of the newborn animals was slow and mortality was very high in the treated group, 50% of the 14-day-old survivors being runts. Only 30% of the treated group were successfully weaned. All runts and many of the larger animals showed motor defects and most of these animals died before weaning. The 29 survivors appeared normal at weaning; they were later test-mated to normal mice of proven fertility. Eight of the 18 males gave sterile plugs and were found to have small testes deficient in germ cells. Two of the eleven females were sterile. Histological examination of the first-generation MMC embryos had shown severe depletion of primordial germ cells up to day 9·5 but about half of the embryos showed good recovery of germ-cell numbers. Migration of primordial germ cells was also retarded.

This study indicates that the mouse embryo can withstand a major disturbance in early development and recover to such an extent that there is little evidence of structural abnormalities. The frequency and nature of abnormalities found at day 13·5 gave no suggestion of the profound effects on postnatal development elicited by MMC given at the pre-organogenesis stage. The authors point out that during the recovery period of accelerated development, the embryo may be more susceptible to damage that would normally be trivial.

[These results emphasize the need to extend teratogenicity studies beyond parturition.]

Fd Cosmet. Toxicol. Vol. 18, p. 751
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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 Cosmetics Toxicology*:

The metabolic fate of [^{14}C]coumarin in baboons. By A. R. Waller and L. F. Chasseaud.

Studies on the metabolism of calcium stearoyl-2-lactylate in the rat, mouse, guinea-pig and man. By J. C. Phillips, C. Topp and S. D. Gangolli.

Studies on liver microsomes of female rats fed purified diets varying in fat content and with and without propyl gallate. By M. M. King and P. B. McCay.

Purification and some properties of chicken liver aflatoxin B_1 reductase. By S. C. G. Chen, R. D. Wei and D. P. H. Hsieh.

Comparative metabolism of zearalenone and transmission into bovine milk. By C. J. Mirocha, S. V. Pathre and T. S. Robison.

Metabolic fate of T-2 toxin in a lactating cow. By T. Yoshizawa, C. J. Mirocha, J. C. Behrens and S. P. Swanson.

Induction par le lindane des monoxygenases microsomales du foie chez le rat: effets d'un jeune de 72 heures. By R. Albrecht, M. A. Pélissier, F. Faudemay, E. Dooh-Priso et J. P. Carreau.

Relationship between the consumption of toxic rice oil and the long-term concentration of polychlorinated biphenyls in the blood of 'Yusho' patients. By H. Hayabuchi, T. Yoshimura, M. Ikeda and Y. Masuda.

Chronic toxicity of 2,3,7,8-tetrachlorodibenzofuran for rhesus macaques. By W. P. McNulty, I. Pomerantz and T. Farrell.

Rat teratology study of orally administered tris-(2,3-dibromopropyl) phosphate. By V. M. Seabaugh, T. F. X. Collins, C. A. Hoheisel, G. W. Bierbower and J. McLaughlin.

The dose-dependent metabolism of [^{14}C]methylene chloride following oral administration to rats. By M. J. McKenna and J. A. Zempel.

Mutagenicity of commercial *p*-phenylenediamine and of an oxidation mixture of *p*-phenylenediamine and resorcinol in *Salmonella typhimurium* TA98. By R. Crebelli, L. Conti, A. Carere and R. Zito.

Size-distribution analysis of respirable particulates in cosmetic aerosols: a methodological comparison. By M. K. Halbert, M. K. Mazumder and R. L. Bond.

Effects of butylated hydroxytoluene and acetylaminofluorene on NADPH-cytochrome *P*-450 reductase activity in rat liver microsomes. By L. E. Rikans, D. D. Gibson, P. B. McCay and M. M. King. (Short paper)

Effect of diet on oestrogen bio-assay in mice. By H. M. Drane, C. N. Hebert and N. Saba. (Short paper)

[*Contents continued*]

Aims and Scope

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

Instructions to Authors

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

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

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

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

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

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

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

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

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

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

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

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

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