Volume 11 Number 3 June 1973

Food and Cosmetics Toxicology

An International Journal published for the
British Industrial Biological Research Association

CONTENTS

Continued on inside back cover]

FCTXAV 11(3) 355–526 (1973)

Pergamon Press OXFORD LONDON NEW YORK PARIS

FOOD AND COSMETICS TOXICOLOGY

An International Journal published for the British Industrial Biological Research Association

Publishing Offices

Pergamon Press Limited, 143 Fore Street, Exeter EX4 3AP, England
Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, New York 10523, U.S.A.

Advertising Office

Pergamon Press Limited, Headington Hill Hall, Oxford OX3 0BW, England

Published bi-monthly

Annual Subscription Rates

For Libraries, Research Establishments and all other mutiple-reader institutions £34 ($85)
Private individuals whose departmental libraries subscribe may obtain this Journal for their personal use at a reduced rate of £6 ($15)
For members of BIBRA £4

Microform Subscriptions and Back Issues

Current subscriptions on microfiche and microfilm, and back files on microfilm as well as back issues in the regular editions of all previously published volumes are available from our sole distributors, Microforms International Marketing Corporation Inc. (MicroMark) at the most convenient address: *Cowper House, Olney, Bucks, England | Fairview Park, Elmsford, New York 10523, U.S.A.*

All subscription enquiries other than those above should be addressed to:

Subscriptions Manager, Pergamon Press Limited, Headington Hill Hall, Oxford OX3 0BW

PERGAMON PRESS LIMITED

HEADINGTON HILL HALL
OXFORD OX3 0BW, ENGLAND

MAXWELL HOUSE, FAIRVIEW PARK
ELMSFORD, NEW YORK 10523, U.S.A.

Fd Cosmet. Toxicol. Vol. 11, p. i. Pergamon Press 1973. Printed in Great Britain

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST

TOXICOLOGY: ABSTRACTS AND COMMENTS

Fd Cosmet. Toxicol. Vol. 11, pp. 355–365. Pergamon Press 1973. Printed in Great Britain

Research Section

Teratology Studies on Food Colourings. Part II. Embryotoxicity of R Salt and Metabolites of Amaranth (FD & C Red No. 2) in Rats

T. F. X. COLLINS and J. MCLAUGHLIN

with the technical assistance of G. C. GRAY

Division of Toxicology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D.C. 20204, USA

(*Received 31 August 1972*)

Abstract—Two metabolites (sodium naphthionate and the R-amino salt) and one intermediate (R salt) of the red dye, amaranth (FD & C Red No. 2), were tested for their effects on rat reproductive processes and progeny development. Dose levels of 15, 30, 100 or 200 mg/kg/day were administered by stomach tube to Osborne–Mendel females during days 0–19 of pregnancy. No adverse clinical signs were observed in any dams, and no adverse effects on implantation were observed after treatment with any compound. A significant increase in the number of litters with multiple resorptions followed treatment with R salt at the 100 and 200 mg/kg dose levels and with sodium naphthionate at 200 mg/kg. The number of offspring with skeletal, sternebral or soft-tissue abnormalities was not dose-related with any of the compounds. Sodium naphthionate at the 100 mg/kg dose level produced a significant increase in the percentage of foetuses with sternebral abnormalities, but did not affect the incidence of soft-tissue abnormalities at any dose level. R-amino salt at 200 mg/kg significantly increased the incidence of litters with one or more foetuses bearing skeletal abnormalities, but did not increase sternebral or soft-tissue abnormalities. The R salt did not affect skeletal, sternebral or soft-tissue development.

INTRODUCTION

The red dye amaranth (FD & C Red No. 2) is very widely used (1·5 million pounds each year) in solid foods, beverages and pharmaceuticals in over 60 countries. The Joint FAO/WHO Expert Committee on Food Additives (1966) determined that the dye could be safely consumed in amounts up to 1·5 mg/kg/day (90 mg/day for an average 60-kg man). The Committee further recommended that studies be made on the colouring's long-term effects on reproductive processes and progeny development. Recent studies by Russian scientists (Shtenberg & Gavrilenko, 1970) in Wistar rats suggested that the dye might have gonadotoxic and possibly embryotoxic effects. A study by Collins & McLaughlin (1972) in Osborne–Mendel rats showed a dose-related effect on the number of live foetuses per litter, the number of litters with one or more and two or more resorptions and the number of litters totally resorbed, indicating a specific foetotoxic effect. However, no effect was seen in the average weight of the foetuses, and no gross terata or skeletal or soft-tissue abnormalities related to the test material appeared in the experimental animals.

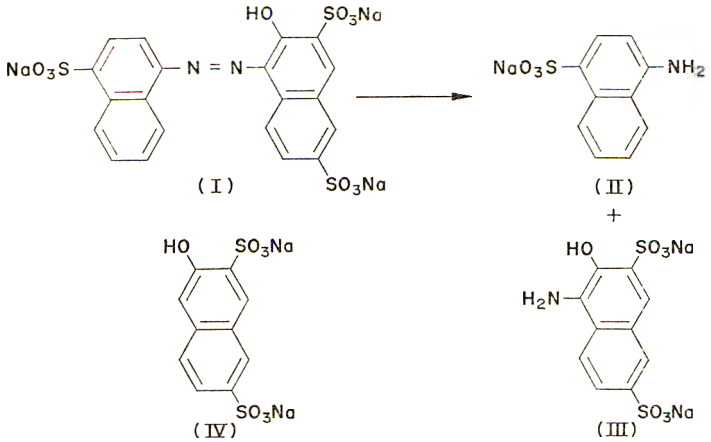


FIG. 1. Structures of FD & C Red No. 2 (I), its metabolites, sodium naphthionate (II) and R-amino salt (III), and its intermediate, R salt (IV).

Radomski & Mellinger (1962) and Roxon, Ryan & Wright (1967) have shown that intestinal bacteria reduce amaranth to the corresponding amines by fission of the azo linkage (see Fig. 1). The two metabolites formed, sodium naphthionate (sodium α-naphthylamine-4-sulphonate) and the R-amino salt (the sodium salt of 1-amino-2-naphthol-3-,6-disulphonic acid), are both excreted in the urine and faeces. Since the specific effects of the metabolites on reproductive processes and progeny development have not been investigated, we conducted a study in Osborne–Mendel rats, and the results are reported here. In addition to the metabolites of amaranth, R salt (2-naphthol-3,6-disulphonic acid sodium salt; Fig. 1), an intermediate and common contaminant of the dye, was investigated.

EXPERIMENTAL

Materials and animals. R salt (2-naphthol-3,6-disulphonic acid sodium salt) was obtained from American Cyanamid Co., Princeton, N.J., and was 38·3% pure (55·7% volatiles, 5·5% sodium chloride, 0·4% sulphate and 0·1% insolubles). Sodium naphthionate (sodium α-naphthylamine-4-sulphonate; 96·7% pure) was obtained from DuPont Chemical Co., Wilmington, Del. R-amino salt (1-amino-2-naphthol-3,6-disulphonic acid sodium salt; 97% pure) was synthesized at the Food and Drug Administration by the Division of Colors and Cosmetics Technology by reduction of FD & C Red No. 1 with sodium hydrosulphite; the product was recrystallized three times. Osborne–Mendel rats, 8–10 wk old, were obtained from the Food and Drug Administration breeding colony.

Procedure. The animals were bred at approximately 5.30 p.m. and the presence of sperm was checked by vaginal smears the following morning (day 0). Females showing a positive indication of sperm were placed on test and the compounds were administered by stomach tube on days 0–19 in doses of 0 (control), 15, 30, 100 or 200 mg/kg/day. All compounds were dissolved in distilled water, the dosage volume being 10 ml/kg body weight. Distilled water alone was given to the controls. On day 20, the animals were killed with chloroform, and the uterus was opened and examined for the presence of resorption sites. Deciduomata, brownish implantation sites without placentas, which failed to develop, were called early deaths, and implantation sites with placentas and with complete but non-viable foetuses of subnormal size or retarded development or in a macerated condition were classed as

late deaths, according to the terminology of Bateman & Epstein (1971) and MARTA (1969). The live foetuses were removed, weighed, sexed and examined for gross external malformations under a dissecting microscope. Corpora lutea were counted under a dissecting microscope. Five foetuses from each litter at each dose level were examined for skeletal anomalies after being fixed in ethyl alcohol, cleared and stained with alizarin red (Dawson, 1926). The remaining foetuses were fixed in Bouin's solution and sectioned according to the method of Wilson & Warkany (1965) in order to detect internal malformations of the soft tissues.

Statistical analysis of results. The data on the number of corpora lutea per dam and the percentage implantation loss for each litter were analysed for statistical significance by the two-tailed *t*-test. The numbers of litters with one or more and two or more resorptions were analysed for statistical significance by the chi-square test. The data on the number of resorptions per litter were transformed utilizing the Freeman–Tukey square-root transformation for Poisson distribution (Mosteller & Youtz, 1961). A two-tailed *t*-test between the control and each dose level was then performed on the transformed data. The data on skeletal, sternebral and soft-tissue abnormalities per litter were transformed by square root transformation and analysed by the two-tailed *t*-test. Data on the percentages of litters with one or more abnormalities were analysed by the chi-square test. Data on the percentages of foetuses with one or more abnormalities in each litter were transformed by using the Freeman–Tukey arc–sine transformation for binomial proportions (Mosteller & Youtz, 1961), followed by a two-tailed *t*-test between the control and each dose level.

RESULTS

Although groups of control animals were maintained for each compound tested, the data have been combined in this paper.

None of the animals died during the treatment period, nor were any adverse clinical signs seen in any control or treated animals. As shown in Table 1, no adverse effects on implantation were seen after treatment with any dose level of any compound. None of the treatments

Table 1. *Implantation data for rats treated orally with metabolites of amaranth or R salt on days 0–19 of pregnancy*

Dose level (mg/kg/day)	No. of dams	Total no. of corpora lutea	Corpora lutea per dam	Total implantations	Implantation loss (%)
0	84	1038	12·36	936	9·83
		Sodium naphthionate			
15	18	219	12·17	202	7·76
30	18	214	11·89	200	6·54
100	20	258	12·90	237	8·14
200	19	238	12·53	214	10·08
		R-amino salt			
15	17	210	12·35	181	13·81
30	17	229	13·47	199	13·10
100	19	248	13·05	223	10·08
200	21	293	13·95	264	9·90
		R salt			
15	18	241	13·39	219	9·13
30	21	260	12·38	226	13·08
100	17	227	13·35	219	3·52*
200	19	243	12·79	222	8·64

The value marked with an asterisk differs significantly from the control value: $*P < 0{\cdot}05$.

led to any decrease in the total number of foetuses per litter, the number of live foetuses per litter or the mean weight of the foetuses (Table 2). The percentage of non-viable implantations was increased at all dose levels of sodium naphthionate and was especially high with 200 mg/kg/day. The percentage of non-viable implantations was increased with doses of 15, 100 and 200 mg R salt/kg/day, but was not altered by any dose level of the R-amino salt.

After treatment of dams with sodium naphthionate, the numbers of litters with one or more resorptions and two or more resorptions were significantly increased at the 200 mg/kg dose level (Table 3). The number of resorptions per litter was increased significantly at the

Table 2. *Foetal survival and weight in rats treated orally with metabolites of amaranth or R salt on days 0–19 of pregnancy*

Dose level (mg/kg/day)	No. of dams	No. of foetuses per litter	Live foetuses per litter	Non-viable implantations		Mean foetal weight (g)
				No.	%	
0	84	11·14	10·42	61	6·52	3·99
			Sodium naphthionate			
15	18	11·22	10·06	21	10·40	3·96
30	18	11·11	10·00	20	10·00	3·80
100	20	11·85	10·55	26	10·97	3·83
200	19	11·26	9·11	41	19·16	3·84
			R-amino salt			
15	17	10·65	9·82	14	7·73	4·09
30	17	11·71	11·47	4	2·01	4·01
100	19	11·74	11·16	11	4·93	3·94
200	21	12·57	11·71	18	6·81	4·01
			R salt			
15	18	12·17	11·06	20	9·13	4·08
30	21	10·76	10·14	13	5·75	4·07
100	17	12·88	10·94	33	15·06	3·93
200	19	11·68	10·00	32	14·41	3·80

highest dose level. Litters were totally resorbed only at the 15 and 200 mg/kg dose levels (one and two litters respectively). With R-amino salt, no significant increases were seen in the number of resorptions per litter or in the number of litters with resorptions (Table 3). After treatment of the dams with R salt, significant increases were seen in the numbers of litters with one or more and two or more resorptions per litter and in the total number of resorptions per litter at dose levels of 100 and 200 mg/kg/day (Table 3).

No external (gross) abnormalities were observed in the controls or after sodium naphthionate treatment (Table 4). In the group given the R-amino salt at 30 mg/kg/day, one foetus with a short tail was observed. In the groups treated with R salt, one exencephalic foetus was seen at the 30 mg/kg dose level and one foetus with a combination of exencephaly, spina bifida and exophthalmia was seen after treatment with 200 mg/kg. No specific skeletal abnormality could be related to treatment with any compound (Table 4). The most frequent skeletal abnormalities were those involving the sternebrae, and poor ossification of bones accounted for the next largest group. Among the kinds of soft-tissue abnormalities, the

kidneys were the organs most commonly affected, showing two kinds of abnormality, namely ectopic kidney and enlargement of the renal pelvis.

With no compound was any dose-relationship seen in the number of skeletal abnormalities (excluding abnormalities in the sternebrae) per litter (Table 5), in the percentage of litters with foetuses bearing skeletal abnormalities or in the percentage of foetuses with one or more skeletal abnormalities. However, significant increases in the percentage of litters with one or more foetuses having skeletal abnormalities appeared with the 30 mg/kg dose level of sodium naphthionate and the 200 mg/kg dose level of R-amino salt.

Table 3. *Incidence of resorption in rats treated orally with metabolites of amaranth or R salt on days 0–19 of pregnancy*

Dosage (mg/kg/day)	Litters with one or more resorptions (ED + LD)		Litters with two or more resorptions (ED + LD)		No. of litters totally resorbed	Resorptions per litter*
	No.*	%	No.*	%		
0	32/84	38·10	14/84	16·67	1	0·73
			Sodium naphthionate			
15	10/18	55·55	6/18	33·33	1	1·16
30	10/18	55·55	4/18	22·22	0	1·11
100	10/20	50·00	7/20	35·00	0	1·30
200	13/19 (<0·05)	68·42	11/19 (<0·001)	57·89	2	2·15 (<0·05)
			R-amino salt			
15	7/17	41·18	5/17	29·41	0	0·82
30	4/17	23·53	0/17	0	0	0·24 (<0·01)
100	5/19	26·32	4/19	21·05	0	0·58
200	11/21	52·38	4/21	19·05	0	0·86
			R salt			
15	8/18	44·44	5/18	27·78	0	1·11
30	8/21	38·10	3/21	14·29	0	0·62
100	13/17 (<0·01)	76·47	9/17 (<0·005)	52·94	0	1·94 (<0·05)
200	13/19 (<0·05)	68·42	10/19 (<0·005)	52·63	0	1·68 (<0·05)

ED = Early deaths LD = Late deaths

*P values are given in parentheses for results differing significantly from controls.

Because of the high rate of sternebral abnormalities in the control animals, a trait inherent in rats (Ward & Readhead, 1970), this type of abnormality was analysed separately (Table 6). Doses of 100 mg sodium naphthionate/kg/day produced a significant increase in the total number of abnormalities, but neither R-amino salt nor R salt caused any significant increase. The percentage of litters with one or more foetuses with sternebral abnormalities was not increased significantly after dosing with any of the compounds. The percentage of foetuses with sternebral defects was significantly increased by treatment with 100 mg sodium naphthionate/kg/day, but not with either of the other two test compounds.

As shown in Table 7, neither the incidence of soft-tissue abnormalities per litter, the percentage of litters with one or more abnormal young nor the percentage of foetuses with soft-tissue abnormalities differed from control values.

Table 4. *Incidence of foetal abnormalities after oral treatment of rats with metabolites of amaranth or R salt on days 0–19 of pregnancy*

	No. of foetuses affected* after treatment with												
	Sodium naphthionate (mg/kg/day)					R-amino salt (mg/kg/day)				R salt (mg/kg/day)			
Abnormality	0	15	30	100	200	15	30	100	200	15	30	100	200
Gross examination													
No. of foetuses examined . . .	872	181	180	211	173	167	193	212	246	199	213	186	190
Exencephaly											1 (0·5)		1 (0·5)
Spina bifida													1 (0·5)
Exophthalmia													1 (0·5)
Short tail							1 (0·5)						
Skeletal abnormalities													
No. of foetuses examined . . .	395	84	85	102	85	77	90	93	117	89	96	87	85
Incomplete sternebrae	163 (41·3)	37 (44·0)	33 (38·8)	48 (47·1)	31 (36·5)	22 (28·6)	40 (44·4)	35 (37·6)	43 (36·8)	30 (33·7)	43 (44·8)	38 (43·7)	39 (45·9)
Bipartite sternebrae	19 (4·8)	10 (11·9)	7 (8·2)	11 (10·8)	5 (5·9)	2 (2·6)		1 (1·1)			1 (1·0)		3 (3·5)
Missing sternebrae	26 (6·6)	6 (7·1)	12 (14·1)	15 (14·7)	3 (3·5)	1 (1·3)	3 (3·3)	5 (5·4)		1 (1·1)	2 (2·1)	5 (5·7)	5 (5·9)
Scrambled sternebrae		3 (3·6)					1 (1·1)	1 (1·1)		1 (1·1)	1 (1·0)	1 (1·1)	
Fused sternebrae	1 (0·3)												1 (1·2)
Poorly ossified ribs	1 (0·3)			1 (1·0)		1 (1·3)	1 (1·1)						
Missing rib	1 (0·3)							1 (1·1					
Poorly ossified pubis	1 (0·3)												
Extra rib		1 (1·2)	2 (2·4)							1 (1·1)	1 (1·0)		
Poorly ossified cranial bones	7 (1·8)	2 (2·4)	3 (3·5)	4 (3·9)	2 (2·4)	2 (2·6)	6 (6·7)	4 (4·3)	6 (5·1)	1 (1·1)	2 (2·1)	2 (2·3)	
Poorly ossified phalanges	4 (1·0)	2 (2·4)		2 (2·0)	1 (1·2)	5 (6·5)		2 (2·2)	2 (1·7)	1 (1·1)		1 (1·1)	
Budded rib	6 (1·5)	1 (1·2)	2 (2·4)		1 (1·2)	1 (1·3)			2 (1·7)	1 (1·1)	3 (3·1)		1 (1·2)
Extra vertebral centrum		1 (1·2)											
Poorly ossified metatarsals									1 (0·9)				
Soft-tissue abnormalities													
No. of foetuses examined . . .	477	97	95	109	88	90	103	119	129	110	117	99	105
Ectopic kidney	14 (2·9)	3 (3·1)	4 (4·2)	4 (3·7)	3 (3·4)	3 (3·3)	5 (4·9)	7 (5·9)	4 (3·1)	3 (2·7)	3 (2·6)	4 (4·0)	7 (6·7)
Hydrocephalus	5 (1·1)	1 (1·0)		1 (0·9)	1 (1·1)			1 (0·8)	1 (0·8)	1 (0·9)	1 (0·9)		2 (1·9)
Diaphragmatic hernia	1 (0·2)												
Haemorrhages	2 (0·4)		2 (2·1						1 (0·8)			1 (1·0)	
Enlarged renal pelvis	4 (0·8)	2 (2·1)	1 (1·1)	1 (0·9)	2 (2·3)	1 (1·1)	4 (3·9)	5 (4·2)		2 (1·8)			1 (1·0)
Incompletely descended testes	1 (0·2)				1 (1·1)			1 (0·8)					
Nasal septum incomplete	3 (0·6)								1 (0·8)			1 (1·0)	
Ventricular septum incomplete	2 (0·4)						1 (1·0)			1 (0·9)	3 (2·6)	2 (2·0)	
Distended ureter	3 (0·6)	3 (3·1)		2 (1·8)				2 (1·6)	3 (2·3)	5 (4·5)	3 (2·6)	1 (1·0)	
Reversed dorsal aorta									1 (0·8)				
Cleft palate												1 (1·0)	

* Incidences expressed as percentages are given in parentheses.

Table 5. *Incidence of skeletal (excluding sternebral) abnormalities in foetuses of rats treated orally with metabolites of amaranth or R salt on days 0–19 of pregnancy*

Test compound	Dose level (mg/kg/day) . . .	Incidence of skeletal abnormalities†				
		0	15	30	100	200
		No. of skeletal abnormalities/litter				
Sodium naphthionate		20/82 (0·24)	7/17 (0·41)	7/18 (0·39)	7/20 (0·35)	4/17 (0·24)
R-amino salt		20/82 (0·24)	9/17 (0·53)	7/17 (0·41)	7/19 (0·37)	11/21 (0·52)
R salt		20/82 (0·24)	4/18 (0·22)	6/21 (0·29)	3/17 (0·18)	1/19 (0·05)
		Proportion of litters with one or more foetuses having skeletal abnormalities				
Sodium naphthionate		14/82 (17·07)	5/17 (29·41)	7/18 (38·89)*	6/20 (30·00)	3/17 (17·65)
R-amino salt		14/82 (17·07)	6/17 (35·29)	5/17 (29·41)	4/19 (21·05)	8/21 (38·10)*
R salt		14/82 (17·07)	4/18 (22·22)	4/21 (19·05)	3/17 (17·65)	1/19 (5·26)
		Proportion of total foetuses having one or more skeletal abnormalities				
Sodium naphthionate		20/395 (5·06)	6/84 (7·14)	7/85 (8·24)	7/102 (6·86)	4/85 (4·71)
R-amino salt		20/395 (5·06)	9/77 (11·69)	7/90 (7·78)	6/93 (6·45)	9/117 (7·69)
R salt		20/395 (5·06)	4/89 (4·49)	6/96 (6·25)	3/87 (3·45)	1/85 (1·18)

†Incidence is expressed as no. affected/total, with percentage in parentheses.
Values marked with an asterisk differ significantly from control values: $*P < 0{\cdot}05$.

Table 6. *Incidence of sternebral abnormalities in foetuses of rats treated orally with metabolites of amaranth or R salt on days 0–19 of pregnancy*

Test compound	Dose level (mg/kg/day)...	Incidence of sternebral abnormalities†				
		0	15	30	100	200
		No. of sternebral abnormalities/litter				
Sodium naphthionate		209/82 (2·55)	56/17 (3·29)	52/18 (2·89)	74/20 (3·70)**	39/17 (2·29)
R-amino salt		209/82 (2·55)	25/17 (1·47)**	44/17 (2·59)	42/19 (2·21)	43/21 (2·05)
R salt		209/82 (2·55)	32/18 (1·78)	47/21 (2·24)	44/17 (2·59)	48·19 (2·53)
		Proportion of litters with one or more foetuses having sternebral abnormalities				
Sodium naphthionate		73/82 (89·02)	17/17 (100)	17/18 (94·44)	20/20 (100)	14/17 (82·35)
R-amino salt		73/82 (89·02)	14/17 (82·35)	17/17 (100)	16/19 (84·21)	18/21 (85·71)
R salt		73/82 (89·02)	13/18 (72·22)	18/21 (85·71)	15/17 (88·24)	16/19 (84·21)
		Proportion of foetuses having one or more sternebral abnormalities				
Sodium naphthionate		205/395 (51·90)	55/84 (65·48)	52/85 (61·18)	73/102 (71·57)**	38/85 (44·71)
R-amino salt		205/395 (51·90)	24/77 (31·17)*	44/90 (48·89)	42/93 (45·16)	43/117 (36·75)*
R salt		205/395 (51·90)	31/89 (34·83)*	45/96 (46·88)	41/87 (47·13)	46/85 (54·12)

†Incidence is expressed as no. affected/total, with percentage in parentheses.
Values marked with asterisks differ significantly from control values: *$P < 0.05$; **$P < 0.01$.

Table 7. *Incidence of soft-tissue abnormalities in foetuses of rats treated orally with metabolites of amaranth or R salt on days 0–19 of pregnancy*

Test compound	Dose level (mg/kg/day)... 0	Incidence of soft-tissue abnormalities* 15	30	100	200
		No. of soft-tissue abnormalities/litter			
Sodium naphthionate	35/79 (0·44)	9/16 (0·56)	7/17 (0·41)	8/19 (0·42)	7/16 (0·44)
R-amino salt	35/79 (0·44)	4/15 (0·27)	10/16 (0·63)	16/18 (0·89)	11/21 (0·52)
R salt	35/79 (0·44)	12/17 (0·71)	10/18 (0·56)	10/16 (0·63)	10/17 (0·59)
		Proportion of litters with one or more foetuses having soft-tissue abnormalities			
Sodium naphthionate	29/79 (36·71)	9/16 (56·25)	6/17 (35·29)	7/19 (36·84)	6/16 (37·50)
R-amino salt	29/79 (36·71)	3/15 (20·00)	8/16 (50·00)	9/18 (50·00)	8/21 (38·90)
R salt	29/79 (36·71)	6/17 (35·29)	8/18 (44·44)	7/16 (43·75)	7/17 (41·18)
		Proportion of foetuses having one or more soft-tissue abnormalities			
Sodium naphthionate	34/477 (7·13)	9/97 (9·28)	6/95 (6·32)	7/109 (6·42)	7/88 (7·95)
R-amino salt	34/477 (7·13)	3/90 (3·33)	9/103 (8·74)	14/119 (11·96)	9/129 (6·98)
R salt	34/477 (7·13)	8/110 (7·27)	8/117 (6·84)	8/99 (8·08)	10/105 (9·52)

*Incidence is expressed as no. affected/total, with percentage in parentheses.

DISCUSSION

In the first paper of this series (Collins & McLaughlin, 1972), amaranth administered to rats was reported to have a dose-related effect on the number of live offspring and on the number of litters with multiple resoprtions. A greatly increased number of late deaths also occurred at the 200 mg/kg level. The study reported here attempted to determine whether the sodium salt of either of the two known metabolites, sodium naphthionate or the R-amino salt, could produce similar results.

Of the two metabolites tested, sodium naphthionate appeared to be more embryotoxic, since it produced increases in the number of non-viable implantations at the highest dose level administered, but neither compound produced definite dose-related embryotoxicity. Neither compound increased the number of late deaths at any level, whereas late deaths followed treatment with amaranth at the 200 mg/kg dose level (Collins & McLaughlin, 1972). When the foetotoxic effects are compared with those of amaranth, which produced dose-related responses in the number of live foetuses per litter, the number of litters with one or more resorptions and the number of litters totally resorbed (Collins & McLaughlin, 1972), neither metabolite appears as embryotoxic as its parent compound, amaranth.

The third compound tested here, R salt, the intermediate and common contaminant of amaranth, produced results similar in severity to those obtained with sodium naphthionate in that it was embryotoxic at dose levels of 100 and 200 mg/kg. In terms of the chemical structure of the compounds, R salt and R-amino salt might have been expected to produce similar effects, as they differ only in a single amine group. However, they did not, suggesting that the amine group may have a definite influence on toxicity. Hence only an incomplete answer to the question of why amaranth is embryotoxic can be offered at this time.

The no-embryotoxic-effect levels for sodium naphthionate and R salt in this experiment were 100 and 30 mg/kg respectively. The R-amino salt failed to produce an embryotoxic effect at any of the levels tested.

Acknowledgements—The author is indebted to Mrs. Elaine V. Collins for editorial assistance in preparing the manuscript and to Mrs. Manjeet Singh for preparing the R-amino salt. Thanks are also due to Mr. D. I. Ruggles and Miss Janet Springer of the Mathematics Division for the statistical analysis of the data.

REFERENCES

Bateman, A. J. & Epstein, S. S. (1971). Dominant lethal mutations in mammals. In *Chemical Mutagens. Principles and Methods for Their Detection.* Vol. 2. Edited by A. Hollaender. p. 541. Plenum Press, New York.

Collins, T. F. X. & McLaughlin, J. (1972). Teratology studies on food colourings. Part I. Embryotoxicity of amaranth (FD & C Red No. 2) in rats. *Fd Cosmet. Toxicol.* **10,** 619.

Dawson, A. B. (1926). A note on the staining of the skeleton of cleared specimens with Alizarin Red S. *Stain Technol.* **1,** 123.

Joint FAO/WHO Expert Committee on Food Additives (1966). Specifications for Identity and Purity and Toxicological Evaluation of Food Colours. WHO/Food Add./66.25, *F.A.O. Nutr. Mtg Rep. Ser.* no. 38B, p. 22.

Mid-Atlantic Reproduction and Teratology Association (MARTA) Committee on Terminology (1969). Teratology and Reproduction Glossary, p. 71.

Mosteller, F. & Youtz, C. (1961). Tables of Freeman–Tukey transformations for the binomial and Poisson distributions. *Biometrika* **4** (3–4), 433.

Radomski, J. L. & Mellinger, T. J. (1962). The absorption, fate and excretion in rats of the water-soluble azo dyes, FD & C Red No. 2, FD & C Red No. 4 and FD & C Yellow No. 6. *J. Pharmac. exp. Ther.* **136,** 259.

Roxon, J. J., Ryan, A. J. & Wright, S. E. (1967). Reduction of water-soluble azo dyes by intestinal bacteria. *Fd Cosmet. Toxicol.* **5,** 367.

Shtenberg, A. I. & Gavrilenko, E. V. (1970). Influence of the food dye amaranth upon the reproductive function and development of progeny in tests on albino rats. *Vop. Pitan* **29** (2), 66.

Ward, R. J. & Readhead, S. M. (1970). The effect of alloxan-induced diabetes on the foetal toxicity of thalidomide, carbutamide and myleran in rats. *Proceedings of the European Society for the Study of Drug Toxicity*. **11**, 151.

Wilson, J. G. & Warkany, J. (1965). *Teratology, Principles and Techniques*. A compendium of lectures and demonstrations given at the First Workshop in Teratology held at the University of Florida, 2–8 February 1964. 1st ed. pp. 263. The University of Chicago Press, Chicago.

Etude tératologique de colorants alimentaires. II. Embryotoxicité du sel R et de métabolites de l'amaranthe (FD & C Red No 2) chez le rat

Résumé—On a étudié les effets de deux métabolites (le naphthionate de sodium et le sel amino-R) et d'un intermédiaire (sel R) du colorant rouge amaranthe (FD & C Red No 2) sur les processus de reproduction du rat et sur le développement de sa descendance. A cette fin on a administré des doses de 15, 30, 100 ou 200 mg/kg/jour par tubage gastrique à des femelles Osborne–Mendel pendant les jours 0 à 19 de la gravidité. Aucun indice clinique de nocivité n'a été observé chez aucune de ces femelles et aucun effet nuisible à l'implantation n'a été constaté après le traitement par un des trois produits essayés. Les traitements au naphthionate de sodium à la dose de 200 mg/kg et au sel R aux doses de 100 et de 200 mg/kg ont été suivis d'une augmentation significative du nombre de portées à résorptions multiples. Le nombre de jeunes présentant des anomalies du squelette, des sternèbres ou des tissus mous n'était pas en rapport avec le dosage, quel que fût le produit testé. Le naphthionate de sodium administré à raison de 100 mg/kg a fait augmenter significativement le pourcentage de foetus présentant des anomalies des sternèbres; à aucune dose il n'a influé sur la fréquence des anomalies des tissus mous. Le sel amino-R à la dose de 200 mg/kg a fait augmenter significativement la fréquence des portées avec un foetus ou plus présentant des anomalies du squelette, mais les anomalies des sternèbres et des tissus mous n'ont pas augmenté. Le sel R n'a pas influé le développement du squelette, des sternèbres ou des tissus mous.

Teratologische Untersuchungen über Lebensmittelfarbstoffe. Teil II. Embryotoxizität von R-Salz und Metaboliten von Amaranth (FD & C Red No. 2) in Ratten

Zusammenfassung—Zwei Metaboliten (Natriumnaphthionat und das R-Aminosalz) und ein Zwischenprodukt (R-Salz) des roten Farbstoffs Amaranth (FD & C Red No. 2) wurden hinsichtlich ihrer Wirkungen auf die Fortpflanzung und die Entwicklung der Nachkommen von Ratten geprüft. Mit der Schlauchsonde wurden Osborne–Mendel-Rattenweibchen während der Tage 0–19 der Trächtigkeit Dosen von 15, 30, 100 oder 200 mg/kg/Tag verabreicht. Bei keinem der Tiere wurden nachteilige klinische Zeichen festgestellt, und es konnten auch keine schädlichen Wirkungen nach Implantation einer der Verbindungen beobachtet werden. Eine signifikante Zunahme der Zahl der Würfe mit mehrfachen Resorptionen folgte der Verabreichung von R-Salz in den Dosierungen 100 und 200 mg/kg und von 200 mg Natriumnaphthionat/kg. Die Zahl der Nachkommen mit Skelett-, Sternebrae- oder Weichteilanomalien war bei keiner der Verbindungen dosisabhängig. Natriumnaphthionat in der Dosierung 100 mg/kg verursachte eine signifikante Zunahme des Prozentsatzes von Foeten mit Sternebrae-Anomalien, beeinflusste aber nicht die Häufigkeit von Weichteilanomalien bei irgendeiner Dosierung. Das R-Aminosalz bei 200 mg/kg erhöte signifikant der Haüfigkeit der Würfe mit einem oder mehreren Foeten mit Skelettanomalien, aber die Sternebrae- und Weichteilanomalien waren nicht vermehrt. Das R-Salz beeinflusste weder die Skelett- und Sternebrae- noch die Weichteilentwicklung.

Fd Cosmet. Toxicol. Vol. 11, pp. 367–374. Pergamon Press 1973. Printed in Great Britain

Short-term Toxicity of Orange G in Pigs

I. F. GAUNT—*Toxicology*
IDA S. KISS and P. GRASSO—*Pathology*
and
S. D. GANGOLLI—*Biological Chemistry*
British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England

(*Received 12 October 1972*)

Abstract—Orange G was given to groups of three male and three female pigs for 112 days at doses of 0 (control), 2·5, 25 or 250 mg/kg/day. No adverse effects were found in pigs given 2·5 mg Orange G/kg/day. At the higher levels, Heinz bodies were found in the erythrocytes, together with anaemia, reticulocytosis, splenomegaly and, at the highest level only, a histologically demonstrable increase in splenic iron. The no-untoward-effect level in this study was 2·5 mg/kg/day.

INTRODUCTION

Orange G (C.I. (1956) no. 16230), also known as Orange GG, D & C Orange No. 3 and Naphthalene Orange Solide GG, is the disodium salt of 1-phenylazo-2-naphthol-6,8-disulphonic acid. It is permitted for use in drugs and cosmetics in the USA but not for use in food, although at present it is permitted for use in food in the UK (Statutory Instrument 1966, no. 1203). A need for further information concerning its biological activity was pointed out by the Food Standards Committee (1964) in its Report on Colouring Matters and by the Joint FAO/WHO Expert Committee on Food Additives (1965), which prepared a specification for Orange G but considered that the data available were insufficient for a toxicological evaluation.

The azo linkage in Orange G is reduced by a homogenate of rat liver (Daniel, 1967) or by bacteria from the rat caecum (Roxon, Ryan & Wright, 1967). The reduction occurs to a greater extent with the soluble fraction from homogenized cultures of *Escherichia coli* or a Proteus species of bacteria than with a similar preparation from rat liver (Ryan, Roxon & Sivayavirojana, 1968). This reduction also occurs in the living animal. Daniel (1962) found aniline, *o*-aminophenol and *p*-aminophenol in the urine of rabbits given oral doses of 0·5 g Orange G/kg. R. Walker, I. F. Gaunt and P. G. Brantom (unpublished information, 1972) found that, in rats given a single dose of 250 mg Orange G/kg body weight, 61% of the dose appeared as *p*-aminophenol in the urine. Aniline in the urine and faeces accounted for 6 and 22% of the dose, respectively. No unchanged colouring was detected in either urine or faeces. It was also found that, in man, following a dose of 20 mg Orange G/kg body weight, 95% of the dose was present in the urine as *p*-aminophenol, 0·5% as aniline and 1·3% as unchanged colouring.

These studies do not indicate the fate of the naphthol moiety. Conjugation is one possible pathway, but the sulphonation of the naphthol in Orange G would render it water-soluble and it is likely to be excreted unchanged, as has been shown for 1-amino-2-naphthol-6-sulphonic acid derived from Orange RN or Sunset Yellow FCF (Daniel, 1962).

The major toxicological finding associated with Orange G is the production of Heinz bodies in the erythrocytes and signs of anaemia with associated erythrophagocytosis and erythropoiesis. Heinz bodies were found in rats given a diet containing 1·0% Orange G for 44 days (Rofe, 1957) and in cats given oral doses of 100 mg/kg for 7 days or 2 mg/kg for 33 days (G. Hecht, unpublished information cited by Deutsche Forschungsgemeinschaft—Farbstoff-Kommission, 1957). Hansen, Wilson & Fitzhugh (1960) fed dietary levels of 0, 0·25, 0·5, 2·0 or 5·0% Orange G to groups of five male and five female rats for 90 days. They reported splenomegaly at all levels, anaemia at all but the lowest level and deaths in those rats given a diet containing 5·0% Orange G.

Gaunt, Wright, Grasso & Gangolli (1971) fed diets containing 0, 50, 500 or 5000 ppm Orange G to rats for 15 wk. They found a no-untoward-effect level of 50 ppm. Heinz bodies were present in a few erythrocytes of the rats given the diet containing 500 ppm of the colouring. At the highest level there was a high incidence of Heinz bodies, together with an anaemia, methaemoglobinaemia, reticulocytosis and splenomegaly.

There was no evidence of carcinogenicity when Orange G was given to mice at 1 mg/day in food for 500 or 700 days (Waterman & Lignac, 1958) or at a rate of 15–20 mg/wk over 5 days/wk for 538 days (Cook, Hewett, Kennaway & Kennaway, 1940). There was no increased tumour production in 25 rats given a diet containing 0·1% Orange G for 400 days or in ten rats given a diet containing 0·2% of the colouring for 245 days (Klinke, unpublished information cited by Deutsche Forschungsgemeinschaft—Farbstoff-Kommission, 1957).

The present paper describes a short-term feeding study in pigs carried out as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. Orange G was supplied through the Food Colours Committee of the Association of British Chemical Manufacturers, now the Chemical Industries Association. It complied with the following specification of the British Standards Institution (1963):

> Dye content, min. 85%; matter volatile at 135°C*, max 10%; matter insoluble in water*, max 0·1%; matter soluble in diisopropyl ether*, max 0·2%; subsidiary dyes, max 1%; primary aromatic amine (as aniline), max 0·02%; chloride and sulphate (as sodium salts)*, max 5%; copper*, max 10 ppm; arsenic*, max 1 ppm; lead*, max 10 ppm; heavy metals (as sulphides)*, producing a colour no more intense than that of the reference standard.

Animals and diet. Pigs of the Large White strain, aged 10 wk, were purchased from a minimal-disease herd. They were caged individually and fed Sow and Weaners Nuts (British Oil and Cake Mills Ltd., London) and water.

Experimental design and conduct. The pigs were obtained as three litters each containing four animals of each sex and these were allocated to four groups so that each group contained one pig of each sex from each litter. These groups were given dose levels of 0 (control),

*By methods of analysis described in BS 3210:1960 (Methods for the Analysis of Water-soluble Coal-tar Dyes Permitted for Use in Foods).

2·5, 25 or 250 mg Orange G/kg body weight/day for 16 wk. The required amount of Orange G was mixed with a small quantity of powdered diet and Golden Syrup (Tate & Lyle Ltd., London), and given before the main feed. In this way the whole of the dose was eaten with the minimum of spillage.

The pigs were weighed initially and then twice weekly during the study. Blood samples were collected from an ear vein of each pig at wk 6 and from the superior vena cava at autopsy. The blood was examined for haemoglobin concentration, packed cell volume and counts of total erythrocytes, reticulocytes, erythrocytes with Heinz bodies and total and differential leucocytes. Methaemoglobin concentrations (Sunderman & Sunderman, 1960) were measured in the blood taken at autopsy. Additional samples of blood were collected from the ear veins of the male pigs on days 0, 2, 6, 9, 13, 15, 20, 23, 27, 30, 34 and 63 and examined for erythrocytes with Heinz bodies and for reticulocytes.

Glutamic–oxalacetic transaminase and glutamic–pyruvic transaminase activities (Karmen, 1955) were measured in serum collected at autopsy.

Urine was collected over a 6-hr period in wk 6 and in the last week of feeding. It was examined for specific gravity, constituents of the centrifuged sediment and for the presence of glucose, ketones, bile salts and blood.

The pigs were killed by exsanguination after intravenous barbiturate and the organs were examined for macroscopic abnormalities. The brain, heart, lungs, liver, spleen, kidneys, stomach, small intestine, caecum, adrenals, gonads, pituitary and thyroid were weighed. Samples of these organs and of trachea, oesophagus, aorta, gall bladder, diaphragm, skeletal muscle, sternum, colon, rectum, pancreas, lymph nodes, sciatic nerve, urinary bladder, ureters, urethra, seminal vesicle, vas deferens, vas efferens and prostate or uterus, fallopian tubes and vagina were preserved in 10% buffered formalin. Paraffin wax sections of these tissues were stained with haematoxylin and eosin for histopathological examination.

RESULTS

There were no abnormalities of behaviour or appearance in any of the pigs. The faeces of pigs on the highest dosage level appeared to be darker than normal and there was a slight orange coloration of the urine. The mean body-weight gain of the Orange G-treated groups did not differ significantly from that of the controls (Table 1).

In the pigs given 250 mg Orange G/kg/day, Heinz bodies developed within the first week of feeding and reached peak incidences by wk 2 of treatment, remaining at slightly lower than peak levels for the remainder of the study (Table 2). A few erythrocytes developed Heinz bodies in the pigs given 25 mg/kg/day but these were not seen until treatment had continued for 5 wk. A marked reticulocytosis accompanied the inclusions at the highest level of treatment but the number of reticulocytes was only slightly increased at the lower treatment level. The other haematological findings (Table 3) were of an anaemia and methaemoglobinaemia in the pigs given 25 or 250 mg Orange G/kg body weight/day.

Apart from the slight orange colour at the highest treatment level, no abnormal constituents were found in the urine of any of the pigs. The serum transaminase levels were similar in treated and control pigs (Table 3). The only dose-related changes in organ weight were found in the spleen (Table 4). There were increases of spleen weight and spleen weight relative to body weight in both sexes given 250 mg/kg/day and in males at the 25 mg/kg/day level. There were also slight increases in liver weight, but these were not statistically significant. The other isolated changes in organ weight were not found consistently in all three

Table 1. *Mean body weights of pigs given 0–250 mg Orange G/kg/day for 16 wk*

Dose level (mg/kg/day)	Body weight (kg) on day no.						Body-weight gain (kg) at 112 days
	0*	21	42	63	84	112	
Males							
0	18·2	20·7	22·8	26·5	29·0	34·3	16·1
2·5	18·0	21·3	23·8	27·0	30·0	35·7	17·7
25	18·0	21·5	24·2	27·2	30·2	35·2	17·2
250	18·7	21·8	23·8	26·7	29·3	34·3	15·6
Females							
0	17·5	21·5	24·3	27·8	32·0	37·2	19·7
2·5	17·3	21·3	24·2	27·2	30·8	35·8	18·5
25	17·5	22·0	25·0	27·7	32·0	37·5	20·0
250	16·8	21·2	24·2	25·8	30·3	35·0	18·2

*First day of feeding.
The figures are means for groups of three pigs.

Table 2. *Incidence of Heinz bodies and reticulocytes in male pigs given 0–250 mg Orange G/kg/day for 16 wk*

Dose level (mg/kg/day)	Incidence on day no.													
	0*	2	6	9	13	15	20	23	27	30	34	42	63	112
Heinz bodies (% of erythrocytes)														
0	0·1	0·2	0·2	0·2	0·2	0	0·1	0·1	0·1	0·3	0·2	0·3	0·2	0·3
2·5	0	0·1	0·1	0·1	0·1	0·2	0·2	0·3	0·1	0·6	1·1	0·2	0·2	0·4
25	0·1	0·2	0·2	0·2	0·3	0·5	0·3	0·5	0·6	0·8	1·4	1·8	1·7	1·8
250	0	0·3	1·7	6·6	20·6	27·3	22·3	19·0	22·6	24·1	25·9	22·2	20·5	23·3
Reticulocytes (% of erythrocytes)														
0	0·9	0·7	0·6	0·8	0·9	0·9	0·5	0·5	0·7	0·4	0·7	1·0	0·4	0·6
2·5	0·8	0·7	0·4	0·4	0·7	0·4	0·4	0·3	0·7	0·9	0·9	0·9	0·8	1·0
25	0·7	0·6	0·3	0·6	0·4	1·0	0·8	0·9	1·0	1·0	1·1	1·7	1·4	1·2
250	0·5	0·6	1·1	2·1	2·5	3·8	2·7	5·1	5·0	3·5	3·0	4·4	2·9	3·1

*First day of feeding.
The figures are means for groups of three pigs.

pigs when these were compared with their litter mate controls and were not seen when the weights were expressed relative to body weight.

There were no abnormal histopathological findings in the pigs given 2·5 or 25 mg Orange G/kg/day. At the highest treatment level (250 mg/kg) there was pigmentation, positive for iron, in the spleen and the Kupffer cells of the liver. This pigmentation was found in all three male animals but in only one female.

DISCUSSION

There were no untoward findings in pigs given 2·5 mg Orange G/kg body weight/day for 16 wk. Heinz bodies were found at the higher treatment levels. They were present in larger

Table 3. *Results of haematological examinations and serum transaminase levels in pigs given 0–250 mg Orange G/kg/day for 6 or 16 wk*

Sex and dose level (mg/kg/day)	Hb (g/100 ml)	MetHb (% of Hb)	PCV (%)	RBC (10^6/mm^3)	HB (% of RBC)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	B	L	M	SGOT (IU)	SGPT (IU)
Wk 6														
Male														
0	15·6	—	45	8·66	0·3	1·0	18·46	27	2	0	70	1	—	—
2·5	14·5	—	42	8·06	0·2	0·9	18·56	25	0	0	73	2	—	—
25	13·6**	—	41	7·61*	1·8*	1·7	19·56	22	1	0	76	1	—	—
250	13·8**	—	41	6·71*	22·2**	4·4**	16·30	28	1	0	69	2	—	—
Female														
0	15·4	—	46	8·72	0·3	0·8	21·21	22	2	0	75	1	—	—
2·5	14·4	—	44	7·97	0·2	0·8	21·31	26	3	1	69	1	—	—
25	14·3	—	44	8·00	1·7*	1·0	17·17	26	2	0	69	3	—	—
250	13·7*	—	43	6·76*	13·8*	4·3*	17·17	25	2	0	71	2	—	—
Wk 16														
Male														
0	11·7	5·25	37	6·90	0·3	0·6	8·92	36	2	0	60	2	14	17
2·5	11·8	5·83	38	6·55	0·4	1·0	11·08	28	2	0	68	2	17	20
25	10·2*	8·14*	33	5·87	1·8	1·2*	10·34	37	2	1	58	2	15	15
250	10·3*	6·68	33	5·47	23·3*	3·1**	18·34	28	3	0	66	3	15	17
Female														
0	12·9	3·96	40	7·41	0·3	0·8	9·56	30	1	0	67	2	18	19
2·5	12·3	3·87	38	7·19	0·5	1·1	9·66	15	2	0	81	2	13	16
25	11·8*	4·20	39	6·12	0·9	1·0	8·83	32	2	0	65	1	11	13
250	10·7**	7·98	36*	5·25**	18·2**	4·2*	12·04	13	5	1	77	4	25	12

Hb = Haemoglobin MetHb = Methaemoglobin PCV = Packed cell volume RBC = Red blood cells HB = Heinz bodies
Reties = Reticulocytes N = Neutrophils E = Eosinophils B = Basophils L = Lymphocytes M = Monocytes
SGOT = Serum glutamic-oxalacetic transaminase SGPT = Serum glutamic-pyruvic transaminase
IU = International units

The figures are means for groups of three pigs. Those marked with asterisks differ significantly (Lord (1947) range test) from those of the controls: *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

Table 4. *Organ weights and relative organ weights of pigs given 0–250 mg Orange G/kg/day for 16 wk*

Sex and dose level (mg/kg/day)	Organ weights													Terminal body weight (kg)
	Brain	Heart	Lung	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals	Gonads	Pituitary†	Thyroid	
Absolute organ weight (g)														
Male														
0	98·0	145·7	229·7	635·3	39·0	91·3	368·3	1149·3	86·3	2·35	134·0	147	2·73	35·7
2·5	99·3	124·7	242·7	772·3	36·7	87·3	375·3	1245·7	68·3*	2·31	139·3	177	2·72	37·0
25	104·7	153·3	275·3	805·3	52·7*	96·3	341·3	1060·0	60·0*	2·53	136·3	157	3·43	37·0
250	89·0	137·0	261·7	794·0	68·3**	101·7	351·0	1112·7	76·0	2·55	112·3*	163	2·35	35·7
Female														
0	92·7	153·0	234·3	868·0	45·3	97·3	378·0	1174·3	67·3	3·01	3·75	213	2·90	40·7
2·5	103·7**	145·3	306·0	750·7	50·0	92·0	373·7	1011·3	82·3*	2·61	3·30	195	3·28	38·8
25	102·0	145·7	263·0	886·3	51·7	98·3	375·0	1138·7	84·7	2·75	3·53	180	3·44	39·5
250	96·7	145·7	230·7	924·0	72·7	104·3	373·3	1021·0	77·3	2·43	4·02	203	3·08	38·0
Relative organ weight (g/kg body weight)														
Male														
0	2·77	4·08	6·48	17·87	1·09	2·58	10·33	31·83	2·45	0·067	3·80	4·11	0·076	
2·5	2·71	3·43	6·59	21·10	0·98	2·37	10·29	34·27	1·86	0·063	3·89	4·82	0·073	
25	2·82	4·16	7·47	21·75	1·43*	2·69	9·23	28·63	1·63	0·069	3·72	4·24	0·092	
250	2·49	3·84	7·28	22·59	1·91**	2·86	9·88	31·65	2·13	0·072	3·15	4·55	0·067	
Female														
0	2·30	3·82	5·80	21·19	1·10	2·39	9·35	28·79	1·67	0·074	0·093	5·23	0·076	
2·5	2·69	3·79	7·85	19·21	1·28	2·37	9·66	26·17	2·13	0·064	0·087	5·21	0·083	
25	2·59	3·70	6·68	22·43	1·32	2·49	9·49	28·69	2·15	0·070	0·090	4·65	0·087	
250	2·56	3·86	6·84	24·46	1·93*	2·76	9·06	27·02	2·04	0·064	0·106	5·36	0·082	

†Weights and relative weights of this organ are expressed in mg and mg/kg body weight respectively.
Values are means for groups of three pigs and those marked with asterisks differ significantly (Lord (1947) range test) from those of the controls: *$P<0·05$; **$P<0·01$.

numbers and developed earlier with the highest dosage level (250 mg/kg) than with the lower (25 mg/kg), with which they did not develop until treatment had continued for 34 days. This longer latent period with lower levels of treatment has been encountered previously with other Heinz body-producing colourings fed to rats (I. F. Gaunt, unpublished information 1972) and has been shown to vary with different colourings (Rofe, 1957). This observation may be associated with the fact that, with low levels of active materials, the erythrocytes may have to be exposed for a substantial proportion of their lifespan before Heinz bodies appear.

It is known that Orange G itself will not form Heinz bodies when it is incubated at concentrations of up to 100 mg/100 ml with erythrocytes (I. F. Gaunt, unpublished information 1972), so the fact that the inclusions are found *in vivo* in the pig suggests that this species, like man and the rat (R. Walker, I. F. Gaunt and P. G. Brantom, unpublished information 1972), is capable of metabolizing the colouring. This suggestion is supported by the fact that no orange colour was seen in the excreta of the pigs given 2·5 or 25 mg/kg and only a slight orange coloration appeared in the urine at the highest level. Although there is no direct evidence, it seems likely that the azo bond in Orange G is reduced by the pig.

The other changes seen after daily doses of 25 or 250 mg Orange G/kg/day were anaemia, splenomegaly, iron deposition in the spleen and liver and reticulocytosis, all of which can be associated with the presence of Heinz bodies. It was shown in rats (Azen & Schilling, 1963) that affected cells were removed by the spleen, but the use of radioactively labelled cells showed that there was also accumulation of the radioactivity in the liver in animals with large numbers of inclusions. These workers demonstrated a linear relationship between the dose of acetylphenylhydrazine given and spleen weight. Rifkind (1965) also demonstrated the role of the spleen in removing cells containing Heinz bodies from the circulation and showed that the breakdown of these cells and their inclusion bodies led to the deposition of iron pigments in the spleen. If sufficient erythrocytes are removed this can lead to an anaemia and a compensatory reticulocytosis. These effects have previously been reported with Orange G in rats (Rofe, 1957; Gaunt *et al.* 1971).

The dose levels at which effects were seen in this study were the same as those in rats (Gaunt *et al.* 1971), in which Heinz bodies were found after the feeding of 500 or 5000 ppm in the diet, levels equivalent to approximately 25 and 250 mg/kg/day. The no-untoward-effect level established in this study, as in the rat, was 2·5 mg/kg/day. Applying the traditional 100-fold safety factor, the acceptable daily intake in man would be 1·75 mg/day for a 70-kg adult or 0·75 mg/day for a 30-kg child. As the true no-effect level in animals is somewhere between 2·5 and 25 mg/kg/day the acceptable daily intake in man is likely to be greater than the values suggested above. Intakes of 1–2 mg/day by man are therefore unlikely to present a toxic hazard. However, this does not take into account the possible additive effects of other Heinz body-producing chemicals, particularly other active colourings which may be present in the food with Orange G.

Acknowledgements—The authors are grateful to the staff of the BIBRA Animal House for maintenance of the experimental animals and to the staff of the Analytical Chemistry, Pathology and Toxicology Departments for technical assistance.

REFERENCES

Azen, E. A. & Schilling, R. F. (1963). Role of the spleen in acetylphenylhydrazine (APH) anemia in rats. *J. Lab. clin. Med.* **62,** 59.

British Standards Institution (1963). Specification for Orange G for Use in Foodstuffs. BS 3612:1963.

Cook, J. W., Hewett, C. L., Kennaway, E. L. & Kennaway, N. M. (1940). Effects produced in the livers of mice by azonaphthalenes and related compounds. *Am. J. Cancer* **40,** 62.
Daniel, J. W. (1962). The excretion and metabolism of edible food colors. *Toxic. appl. Pharmac.* **4,** 572.
Daniel, J. W. (1967). Enzymic reduction of azo food colourings. *Fd Cosmet. Toxicol.* **5,** 533.
Deutsche Forschungsgemeinschaft—Farbstoff-Kommission (1957). Mitteilung 6. 2. Auflage. *Toxikologische Daten von Farbstoffen und ihre Zulassung für Lebensmittel in verschiedenen Ländern.* p. 9. Franz Steiner Verlag GmbH, Wiesbaden.
Food Standards Committee (1964). Report on Colouring Matters. HMSO, London.
Gaunt, I. F., Wright, M., Grasso, P. & Gangolli, S. D. (1971). Short-term toxicity of Orange G in rats. *Fd Cosmet. Toxicol.* **9,** 329.
Hansen, W. H., Wilson, Diana C. & Fitzhugh, O. G. (1960). Subacute oral toxicity of ten D & C coal tar colours. *Fedn Proc. Fedn Am. Socs exp. Biol.* **19,** 390.
Joint FAO/WHO Expert Committee on Food Additives—Eighth Report (1965). Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation: Food Colours and some Antimicrobials and Antioxidants. *Tech. Rep. Ser. Wld Hlth Org.* **309.**
Karmen, A. (1955). A note on the spectrophotometric assay of glutamic-oxalacetic transaminase in human blood serum. *J. clin. Invest.* **34,** 131.
Lord, E. (1947). The use of range in place of standard deviation in the *t*-test. *Biometrika* **34,** 41.
Rifkind, R. A. (1965). Heinz body anaemia: An ultrastructural study. II. Red cell sequestration and destruction. *Blood* **26,** 433.
Rofe, P. (1957). Azo dyes and Heinz bodies. *Br. J. ind. Med.* **14,** 275.
Roxon, J. J., Ryan, A. J. & Wright, S. E. (1967). Reduction of water-soluble azo dyes by intestinal bacteria. *Fd Cosmet. Toxicol.* **5,** 367.
Ryan, A. J., Roxon, J. J. & Sivayavirojana, A. (1968). Bacterial azo reduction: A metabolic reaction in mammals. *Nature, Lond.* **219,** 854.
Sunderman, F. W. & Sunderman, F. W. Jr. (1960). *Hemoglobin, Its Precursors and Metabolites.* p. 53, Lippincott, Philadelphia, USA.
Waterman, N. & Lignac, G. O. E. (1958). The influence of the feeding of a number of food colours on the occurrence of tumours in mice. *Acta physiol. pharmac. néerl.* **7,** 35.

Toxicité à court terme de l'Orange G chez le porc

Résumé—Des groupes de trois porcs mâles et de trois porcs femelles ont consommé de l'Orange G pendant 112 jours à raison de 0 (témoins), 2,5, 25 ou 250 mg/kg/jour. On n'a observé aucun effet nocif chez les porcs qui recevaient la dose de 2,5 mg. Chez ceux qui recevaient les doses plus élevées on a constaté la présence de corps de Heinz dans les érythrocytes, ainsi que de l'anémie, de la réticulocytose et de la splénomégalie. Une augmentation histologiquement établie de la teneur en fer de la rate n'a été constatée que pour la dose la plus élevée. Le seuil d'indifférence déterminé dans cette étude se situait à 2,5 mg/kg/jour.

Kurzzeit-Toxizität von Orange G bei Schweinen

Zusammenfassung—Orange G wurde an Gruppen von drei männlichen und drei weiblichen Schweinen 112 Tage lang in Dosen von 0 (Kontrolle), 2,5, 25 oder 250 mg/kg/Tag verfüttert. Keine nachteiligen Wirkungen wurden bei Schweinen gefunden, die 2,5 mg Orange G/kg/Tag erhielten. Bei den höheren Konzentrationen wurden Heinzsche Körperchen in den Erythrocyten zusammen mit Anämie, Reticulocytose, Splenomegalie und, nur bei der höchsten Konzentration, einer histologisch nachweisbaren Zunahme des Eisens in der Milz gefunden. Die von nachteiligen Wirkungen freie Konzentration betrug bei dieser Untersuchung 2,5 mg/kg/Tag.

Fd Cosmet. Toxicol. Vol. 11, pp. 375–382. Pergamon Press 1973. Printed in Great Britain

Long-term Toxicity Studies of Chocolate Brown FB in Mice

I. F. GAUNT and P. G. BRANTOM—*Toxicology*

and

P. GRASSO and IDA S. KISS—*Pathology*

British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England

(*Received 12 October 1972*)

Abstract—Groups of mice were fed on diets containing 0 (control), 300, 1000, 3000 or 10,000 ppm Chocolate Brown FB for 80 wk. There were no dose-related effects on body-weight gain, haematology or organ weights. There was no evidence of any increase in tumour production in mice given Chocolate Brown FB. Pigment was found in the Kupffer cells of the liver, the phagocytic cells of the lymph nodes and spleen, the alveolar macrophages of the lungs and the epithelial cells of the small intestine in mice fed on a diet containing 10,000 ppm Chocolate Brown FB. The no-untoward-effect level of Chocolate Brown FB established in this study was 3000 ppm (equivalent to an intake of approximately 430 mg/kg/day).

INTRODUCTION

Chocolate Brown FB is listed in the 1956 Colour Index but has no number or specification. It is prepared by diazotizing naphthionic acid and coupling it with morin and maclurin (2′,3,4′,5,7-pentahydroxyflavone and pentahydroxybenzophenone respectively). It is at present included in the UK permitted list (The Colouring Matter in Food Regulations 1966, Statutory Instrument 1966, no. 1203), but reports of the Food Standards Committee (1964) and the Joint FAO/WHO Expert Committee on Food Additives (1965) stressed the need for further evidence of safety-in-use if Chocolate Brown FB were to continue in use as a food colouring.

Studies *in vitro* by Fore, Walker & Goldberg (1967) showed that Chocolate Brown FB was degraded by rat-liver homogenate but at a lower rate (1 μg/mg protein/hr) than Brown FK (5 μg/mg protein/hr) or Chocolate Brown HT (2 μg/mg protein/hr). Gaunt, Hall, Farmer & Fairweather (1967) found that orally intubated daily doses of 2000 mg/kg in rats or 1000 mg/kg in mice were tolerated for 3 wk without death. In a short-term feeding study, these authors established a no-effect level of 3000 ppm of the diet of rats for 90 days (approximately 150 mg/kg/day). At dietary levels of 10,000 and 30,000 ppm, pigment was found in cells of the alimentary canal, lymph nodes and renal tubules. Additionally pigment was present in the Kupffer cells of the liver at 30,000 ppm. This pigment was thought to represent an uptake by the cells of the reticulo-endothelial system of the colouring or a metabolite bound to protein. Gaunt, Brantom, Grasso, Creasey & Gangolli (1972) carried out a 2-yr feeding study in rats at the same dietary levels as those used in the 90-day study.

Again the only finding attributable to treatment was pigment deposition, but in this case it was also seen at the 3000 ppm dietary level. A no-untoward-effect level was established at 1000 ppm of the diet for 2 yr (approximately 50 mg/kg/day).

Continuing the investigation of the safety-in-use of Chocolate Brown FB, an 80-wk feeding study in mice was carried out as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. The sample of Chocolate Brown FB used in this study was supplied through the Food Colours Committee of the Association of British Chemical Manufacturers (now the Chemical Industries Association). It was stated to contain 81·8% dye, 6·0% volatile material (loss at 135°C), 0·9% ether extractable material, 0·05% water-insoluble material, 11·3% chlorides and sulphates (expressed as sodium salts), 0·5 ppm arsenic, 2·0 ppm lead and 14·0 ppm copper.

Animals and diet. The animals used in this study were SPF-derived mice of the CFW strain obtained from Carworth Farm USA. They were kept in an animal room maintained at 21 ± 1°C with a relative humidity of 50–60%, and were caged in groups of 15. Diet (reground Oxoid pasteurized breeding diet for rats and mice) and water were provided *ad lib.*

Experimental design and conduct

Groups of 30 male mice (body weight 21–30 g) and 30 females (body weight 17–25 g) were fed diets containing 300, 1000, 3000 or 10,000 ppm Chocolate Brown FB for 80 wk. The control group consisted of 60 male and 60 female mice fed the basic diet for the same period.

The animals were observed for any abnormalities of condition or behaviour and were weighed on alternate weeks throughout the study. Any mouse which appeared to be ill was isolated, to be returned to its cage if its condition improved or killed if its condition deteriorated. An autopsy was conducted on all animals unless this was precluded by advanced autolysis. The animals were killed by exsanguination from the aorta under barbiturate anaesthesia. At autopsy any macroscopic abnormalities were noted and, in mice surviving to 80 wk, the brain, heart, liver, spleen, kidneys and testes were weighed. Samples of these organs and of salivary gland, thyroid, thymus, adrenals, lymph nodes, pancreas, pituitary, ovaries, uterus, urinary bladder, lungs, stomach, duodenum, ileum, colon, caecum, rectum, spinal cord, skeletal muscle and any other tissue that appeared to be abnormal were preserved in 10% buffered formalin. Paraffin wax sections of these tissues were stained with haematoxylin and eosin. Histopathological examination was carried out on all tissues from the control mice and from those fed on the 10,000 ppm diet. At the lower levels, the examination was confined to the liver and kidney and any tissues seen to be abnormal at autopsy. Sections of liver and spleen from selected mice from the highest treatment level were stained with Sudan Black B, by the periodic acid-Schiff procedure, by Schmorl's and Fontana's techniques for reducing substances and by Perls' technique for iron (Pearse, 1968).

Blood samples were taken from a caudal vein of ten male and ten female mice from the control and top two levels (3000 and 10,000 ppm) at 13, 26 and 52 wk and from the aorta of all surviving mice at the termination of the study. They were examined for haemoglobin content and packed cell volume and counts were made of erythrocytes, reticulocytes and total and differential leucocytes.

RESULTS

There were deaths in most groups during the study (Table 1), but there were no statistically significant differences between treated and control groups in terms of death rate or total deaths by wk 80.

Table 1. *Cumulative death record of mice fed Chocolate Brown FB at 0–10,000 ppm in the diet for 80 wk*

Duration of treatment (wk)	Dietary level (ppm) . . .	Cumulative mortality									
		Males					Females				
		0	300	1000	3000	10,000	0	300	1000	3000	10,000
8		1	0	0	0	0	0	0	0	0	0
16		2	0	0	0	0	1	0	0	0	0
24		3	0	0	0	0	1	0	0	1	0
32		5	1	0	0	1	3	0	0	1	0
40		6	1	0	0	1	3	0	0	1	0
48		6	3	2	0	1	4	1	0	2	0
56		6	3	2	0	2	4	2	0	3	0
64		8	4	3	1	3	4	2	0	3	0
72		11	6	6	2	6	7	4	0	3	0
80		14	9	6	4	7	8	5	0	4	0

Deaths shown are from groups of 30 animals in the case of those fed Chocolate Brown FB and of 60 in control groups.

No adverse effects on body-weight gain were seen in females (Table 2). Males fed a dietary level of 300 ppm Chocolate Brown FB gained less weight than the controls, and at autopsy the mean body weight was about 7% less than that of the controls (Table 3). There was a similar difference from controls in the mean terminal body weight of males given a diet containing 10,000 ppm.

Table 2. *Mean values of body weight and body-weight gain of mice fed Chocolate Brown FB at 0–10,000 ppm in the diet for 80 wk*

Dietary level (ppm)	Body weight (g) at wk						Weight gain (g) at wk 78†
	0*	13	25	52	64	78†	
				Males			
0	26·4	33·6	37·1	40·1	41·7	40·2	13·8
300	28·8	33·6	37·9	38·8	40·5	38·3	9·5
1000	26·6	33·0	40·2	41·5	42·9	41·0	14·4
3000	26·9	33·4	39·3	40·6	42·1	39·1	12·2
10,000	26·6	33·5	39·0	41·0	41·6	38·8	12·2
				Females			
0	21·7	28·2	30·4	33·7	34·4	32·7	11·0
300	22·2	30·8	33·5	36·0	36·5	34·8	12·6
1000	22·8	31·6	33·9	34·9	35·9	34·2	11·4
3000	21·3	28·6	33·7	33·9	35·0	34·8	13·5
10,000	23·0	28·9	32·3	34·3	35·0	34·3	11·3

*First day of feeding.
†Recordings of body weight were not made after wk 78.
The figures are the means for all surviving mice (initially 30 in the treated groups and 60 in the controls).

Table 3. *Absolute and relative organ weights of mice fed Chocolate Brown FB at 0–10,000 ppm in the diet for 80 wk*

Sex and dietary level (ppm)	No. of mice examined	Organ						Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Gonads	
Absolute organ weight (g)								
Male								
0	43	0·44	0·20	1·76	0·12	0·56	0·17	36·1
300	19	0·44	0·21	1·70	0·14	0·58	0·17	33·5*
1000	21	0·44	0·20	1·60	0·11	0·57	0·17	35·9
3000	26	0·44	0·21	1·77	0·11	0·58	0·17	35·2
10,000	22	0·44	0·21	1·67	0·13	0·59	0·17	34·0
Female								
0	51	0·43	0·14	1·41	0·12	0·38	—	28·9
300	25	0·43	0·15	1·39	0·11	0·39	—	30·2
1000	29	0·43	0·15	1·41	0·12	0·37	—	29·9
3000	23	0·43	0·16	1·44	0·15	0·40	—	29·4
10,000	27	0·42	0·14	1·33	0·12	0·38	—	29·8
Relative organ weight (g/100 g body weight)								
Male								
0	43	1·21	0·56	4·86	0·34	1·56	0·47	
300	19	1·30	0·63	5·09	0·41	1·72**	0·50	
1000	21	1·23	0·55	4·45	0·30	1·59	0·46	
3000	26	1·26	0·60	5·03	0·30	1·65	0·47	
10,000	22	1·30	0·63	4·90	0·38	1·74**	0·49	
Female								
0	51	1·47	0·49	4·89	0·43	1·30	—	
300	25	1·43	0·49	4·60	0·37	1·28	—	
1000	29	1·42	0·51	4·72	0·41	1·23	—	
3000	23	1·45	0·54	4·90	0·49	1·35	—	
10,000	27	1·43	0·46	4·47	0·40	1·27	—	

Values are means of the numbers of animals shown and those marked with asterisks differ significantly (Student's *t* test) from those of controls: $*P<0·05$; $**P<0·01$.

There were no statistically significant differences between the organ weights of the treated animals and those of the controls (Table 3). However, when these weights were expressed relative to body weight, the figures for male mice fed the diets containing 300 or 10,000 ppm Chocolate Brown FB were all slightly higher than those for control animals. The difference was statistically significant in the case of the kidney.

There were no statistically significant differences between treated and control groups in the results of the haematological examination at wk 80 and no adverse effects at wk 13 (Table 4). There were reductions in the erythrocyte count, packed cell volume and haemoglobin concentration at wk 26 and 52 but these amounted to less than 10% of the control values. At wk 26 the reductions were not dose-related and the only significant effect was a reduction in haemoglobin level in females fed 3000 ppm. At wk 52 the effects were confined to the highest treatment level.

On histological examination, brown pigment was found in the Kupffer cells of the liver and the phagocytic cells of the spleen in both sexes of mice fed diets containing 10,000 ppm Chocolate Brown FB. Some 60–75% of the females were affected compared with only 17% of the livers and 3% of the spleens of the males. Pigment was also found in the epithelial

cells of the small intestine, the phagocytic cells of the lymph nodes and the alveolar macrophages of the lungs in a few females. The pigment did not stain with any of the staining methods employed.

Table 4. *Results of haematological examinations of mice fed Chocolate Brown FB at 0–10,000 ppm in the diet for 80 wk*

Sex and dietary level (ppm)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Wk 13										
Male										
0	10	14·8	48	7·85	1·0	12·5	14	0	84	2
3000	10	16·3*	51	8·93*	0·7	10·6	27	0	71	2
10,000	10	14·8	46	7·39	1·0	12·2	10	1	87	2
Female										
0	10	14·4	50	8·76	1·0	12·7	11	0	87	2
3000	10	15·8**	51	8·87	0·6	14·1	11	0	87	2
10,000	10	16·4***	52	8·65	1·0	10·6	11	1	86	2
Wk 52										
Male										
0	10	13·5	41	7·48	2·3	13·2	31	0	67	2
3000	10	13·4	41	7·22	2·4	11·1	26	0	72	2
10,000	10	13·2	38**	6·84	3·7	10·7	21	0	77	2
Female										
0	10	15·4	47	8·57	2·8	9·3	12	1	86	1
3000	10	15·9	53	8·00	1·5	11·4	15	0	84	1
10,000	10	14·0**	48	7·67**	2·0	11·2	15	1	82	2
Wk 80										
Male										
0	43	12·6	38	7·34	6·7	3·8	36	1	62	2
300	19	12·8	39	7·29	5·4	3·3	43	0	55	2
1000	21	12·9	38	7·52	8·2	3·8	42	0	55	3
3000	26	13·0	39	7·38	4·8	3·7	39	1	58	2
10,000	22	12·9	40	7·22	7·9	3·9	38	1	59	2
Female										
0	51	14·0	43	7·78	4·6	4·1	40	1	58	1
300	25	13·6	41	7·63	4·5	3·8	43	1	55	1
1000	29	14·2	43	8·02	3·2	3·9	43	2	54	1
3000	23	13·2	43	7·77	4·5	3·9	45	2	51	2
10,000	27	14·3	43	8·21	3·3	4·3	39	2	57	2

Hb = Haemoglobin PCV = Packed cell volume RBC = Erythrocyte count
Retics = Reticulocytes N = Neutrophils E = Eosinophils
L = Lymphocytes M = Monocytes

Values are means of the numbers of mice shown and those marked with an asterisk differ significantly (Student's *t* test) from those of controls: *$P<0{\cdot}05$; **$P<0{\cdot}01$; ***$P<0{\cdot}001$.

Basophils did not account for more than 0·5% of the leucocytes in any group.

The incidences of the other histopathological findings are shown in Table 5. There were chronic inflammatory or degenerative changes in the lung, liver, spleen, kidney and urinary bladder, but treatment with Chocolate Brown FB had no influence on the incidence or severity of these lesions.

Table 5. *Histological abnormalities found in mice fed Chocolate Brown FB at 0–10,000 ppm in the diet for 80 wk*

Organ and histological finding	Dietary level (ppm) . . .	Incidence of histological abnormality*									
		Males					Females				
		0	300	1000	3000	10,000	0	300	1000	3000	10,000
	No. of mice examined . . .	53	29	29	29	29	60	30	30	26	30
Lesions other than tumours											
Lung											
Chronic inflammatory infiltration		7	2	2	1	1	15	5	6	1	3
Liver											
Chronic degeneration		2	3	1	7	1	4	1	1	0	1
Spleen											
Chronic degeneration		0	0	1	0	2	1	0	0	0	0
Kidney											
Chronic inflammatory infiltration		11	7	0	6	1	4	3	0	0	0
Bladder											
Chronic inflammatory infiltration		1	0	0	0	1	0	0	0	0	0
Tumours											
Lung											
Adenoma		13	2	5	8	8	19	13	9	6	4
Ovary											
Benign cysts		—	—	—	—	—	5	2	2	0	2
Uterus											
Fibromyoma		—	—	—	—	—	1	1	0	1	0
Mammary											
Adenoma		—	—	—	—	—	0	0	0	0	1
Carcinoma		—	—	—	—	—	1	0	2	0	0
Non-specific											
Generalized lymphoma		0	0	0	0	0	1	1	1	0	0
Subcutaneous fibroma		1	1	2	1	0	0	1	0	0	0

*The figures represent the numbers of mice affected out of the numbers of mice shown.

Tumours were found in the lung, ovary, uterus, mammary glands, lymphoid tissue and subcutaneous tissue. With the exception of the one mammary adenoma found in a female from the 10,000 ppm group, the tumours occurred with similar frequency in both control and treated mice.

DISCUSSION

The feeding of Chocolate Brown FB in the diet at levels up to 10,000 ppm had no effect on the mortality of mice. The lower body-weight gain and terminal body weight of males fed 300 ppm Chocolate Brown FB in the diet were not thought to represent a toxic effect, as similar differences were not found in animals fed higher levels of the colouring and were not repeated in females at the same, or higher, levels of feeding.

The higher relative weights of all organs from male mice fed dietary levels of 300 and 10,000 ppm Chocolate Brown FB were due to the slightly lower terminal body weights of

these animals. As the absolute weights of these organs were well within the range of those from control animals, the differences found were not considered to be of any toxicological significance.

The slightly decreased haemoglobin concentration and erythrocyte counts seen at wk 52 in both sexes fed 10,000 ppm Chocolate Brown FB in the diet indicated a slight anaemia at this stage of treatment. This effect did not progress, no differences being seen between treated and control animals at wk 80.

The deposition of pigment in the phagocytic cells of the liver, spleen, intestine, lymph nodes and lungs of mice fed at the highest level is consistent with the findings in rats fed high dietary levels of this colouring (Gaunt *et al.* 1967 & 1972), although in rats, pigment was also found in cells of the kidney tubules. The fact that the pigment did not stain with any of the methods employed suggests that it was not lipofuscin or haemosiderin.

It has previously been suggested (Gaunt *et al.* 1967 & 1972) that the pigment seen may represent an uptake of protein-bound material by cells of the reticulo-endothelial system. This is unlikely to be Chocolate Brown FB itself, as it does not bind to any marked extent with serum proteins (Gangolli, 1969). However, the azo bonds in Chocolate Brown FB can be reduced (Fore *et al.* 1967) and it is speculated that the amine metabolites are protein-bound and could be taken up as described.

No other histopathological changes were associated with the pigment deposition in these tissues and thus the finding of pigment alone may not have any pathological significance. However, if material is stored in cells and cannot readily be removed, this may represent an undesirable burden with possible, but as yet undefined, effects on their function. Such a prolonged storage appears to be the case with the pigment found after Chocolate Brown FB treatment in rats. A group of rats was fed a diet containing 10,000 ppm Chocolate Brown FB for 3 months and then returned to a diet free of colouring. Animals killed 3 and 6 months after the cessation of treatment still had appreciable amounts of histologically visible pigment in the phagocytic cells of the liver and spleen (P. G. Brantom, M. G. Wright and I. F. Gaunt, unpublished observations 1971).

The feeding of diets containing Chocolate Brown FB did not influence the incidence or severity of the chronic inflammatory and degenerative changes normally seen in mice of this age. Similarly, the tumour incidence was not affected by the treatment and the tumours seen were of types which have been shown to occur spontaneously in mice (Cloudman, 1956; Tucker & Baker, 1967). The single mammary adenoma, which occurred in a mouse fed on a diet containing Chocolate Brown FB without any similar finding in the controls, could not be attributed to treatment, as a similar incidence of this tumour has been reported in untreated mice (Tucker & Baker, 1967).

The results of this study show no evidence that Chocolate Brown FB has any carcinogenic potential in mice when fed at levels up to 10,000 ppm in the diet. A no-untoward-effect level for Chocolate Brown FB in mice has been established at 3000 ppm in the diet for 80 wk with effects of doubtful significance at 10,000 ppm. This no-untoward-effect level is equivalent to an intake of approximately 430 mg/kg/day. Although the actual intake of this colouring in man is unknown, it is unlikely to exceed 5 mg/day (0·7 mg/kg/day for a 70-kg adult), and the no-effect level in mice is thus approximately 600 times the expected maximum intake in man.

Acknowledgements—The authors wish to thank Mrs. G. Wexler for assistance with the statistical analyses in this study. They are also grateful to the staff of the BIBRA Animal House for maintenance of the experimental animals and to the staff of the Pathology and Toxicology Departments for technical assistance.

REFERENCES

Cloudman, A. M. (1956). Spontaneous neoplasms in mice. In *Biology of the Laboratory Mouse.* Edited by G. D. Snell. p. 168. Dover Publications, Inc., New York.

Food Standards Committee (1964). Report on Colouring Matters. HMSO, London.

Fore, H., Walker, R. & Golberg, L. (1967). Studies on Brown FK. II. Degradative changes undergone *in vitro* and *in vivo*. *Fd Cosmet. Toxicol.* **5,** 459.

Gangolli, S. D. (1969). Studies on the action of certain surface active substances on the cell membrane. Ph.D. Thesis, London University.

Gaunt, I. F., Brantom, P. G., Grasso, P., Creasey, Margaret & Gangolli, S. D. (1972). Long-term feeding study on Chocolate Brown FB in rats. *Fd Cosmet. Toxicol.* **10,** 3.

Gaunt, I. F., Hall, D. E., Farmer, Madge & Fairweather, F. A. (1967). Acute (mouse and rat) and short-term (rat) toxicity studies on Chocolate Brown FB. *Fd Cosmet. Toxicol.* **5,** 159.

Joint FAO/WHO Expert Committee on Food Additives—Eighth Report (1965). Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation: Food Colours and some Antimicrobials and Antioxidants. *Tech. Rep. Ser. Wld Hlth Org.* **309.**

Pearse, A. G. E. (1968). *Histochemistry Theoretical and Applied.* 3rd ed. J. & A. Churchill, London.

Tucker, Mary J. & Baker, S. B. de C. (1967). Diseases of specific pathogen-free mice. In *Pathology of Laboratory Rats and Mice.* Edited by E. Cotchin and F. J. C. Roe. p. 787. Blackwell Scientific Publications, Oxford.

Etude de la toxicité à long terme du Brun Chocolat FB chez la souris

Résumé—Des groupes de souris ont consommé du Brun Chocolat FB pendant 80 semaines, à raison de 0 (témoins), 300, 1000, 3000 ou 10 000 ppm du régime. On n'a pas constaté d'effets proportionnels au dosage sur le gain de poids, sur l'hématologie et sur le poids des organes ni d'indices d'une augmentation quelconque de la fréquence de tumeurs chez les souris qui avaient consommé le produit. Le pigment a été trouvé dans les cellules de Kupffer du foie, les cellules phagocytiques des ganglions lymphatiques et de la rate, les macrophages alvéolaires des poumons et les cellules épithéliales de l'intestin grêle des souris qui avaient été soumises au régime à 10 000 ppm de Brun Chocolat FB. Le seuil d'indifférence déterminé dans cette étude est de 3000 ppm, ce qui correspond à une consommation d'environ 430 mg/kg/jour de Brun Chocolat FB.

Langzeit-Toxizitätsuntersuchungen mit Chocolate Brown FB an Mäusen

Zusammenfassung—Gruppen von Mäusen erhielten 80 Wochen lang Futter mit 0 (Kontrolle), 300, 1000, 3000 oder 10 000 ppm Chocolate Brown FB. Es traten keine dosisabhängigen Wirkungen auf die Körpergewichtszunahme, Hämatologie oder Organgewichte auf. Es gab kein Anzeichen einer Zunahme der Tumorhäufigkeit bei Mäusen, die Chocolate Brown FB erhielten. Pigment wurde in den Kupfferschen Zellen der Leber, den Phagocytenzellen der Lymphknoten und der Milz, den alveolaren Makrophagen der Lunge und den Epitelzellen des Dünndarms von Mäusen gefunden, die Futter mit 10 000 ppm Chocolate Brown FB erhalten hatten. Die von nachteiligen Wirkungen freie Konzentration von Chocolate Brown FB, die bei dieser Untersuchung festgestellt wurde, betrug 3000 ppm (entsprechend einer Aufnahme von etwa 430 mg/kg/Tag).

Fd Cosmet. Toxicol. Vol. 11, pp. 383–389. Pergamon Press 1973. Printed in Great Britain

Influence de la Teneur en Protéines du Régime sur Quelques Effets du Bordeaux S et du Jaune de Beurre chez le Rat

R. ALBRECHT, PH. MANCHON et R. LOWY

Laboratoire de Biologie du Conservatoire National des Arts et Métiers, 75-Paris, France

(*Reçu le 26 juin 1972*)

Résumé—Nous avons étudié l'influence d'une réduction des protéines alimentaires chez des rats témoins et chez ceux qui sont traités soit par un additif alimentaire (Bordeaux S), soit par un hépatocancérogène (Jaune de Beurre). Les animaux traités par le Bordeaux S ne réagissent pas différemment des témoins à la carence protéique. La réduction des protéines alimentaires paraît accentuer, en revanche, l'abaissement d'activité de la phosphatase du 6-phosphorylglucose dû au Jaune de Beurre; elle empêche aussi l'induction de la déshydrogénase du 6-phosphorylglucose du à l'hépatocancérogène. Chez tous les animaux, le régime hypoprotéique abaisse spécifiquement l'activité de la *N*-déméthylase de l'aminopyrine. Il est possible que le métabolisme microsomal *in vitro* des substances exogènes soit différent selon la nature de la solubilité du substrat.

INTRODUCTION

Dans un article précédent (Albrecht, Manchon, Keko-Pinto et Lowy, 1973), nous avons étudié l'évolution au cours du temps de quelques effets du Bordeaux S (Amaranthe; C.I. (1956) no. 16185) et du Jaune de Beurre (DAB; C.I. (1956) no. 11020) chez le rat.

Une idée qui commence à se répandre, est qu'un traitement sans effet notable sur un individu réputé normal et bien nourri, pourrait avoir des répercussions nettes sur un sujet traité par ailleurs ou présentant des déficiences physiopathologiques. Il nous a donc paru intéressant d'étudier l'influence d'une réduction des protéines alimentaires chez des rats témoins et chez ceux qui sont traités par le Bordeaux S ou le Jaune de Beurre. On sait, en effet, que le pouvoir de détoxication de l'organisme est perturbé lorsque l'apport protéique des régimes est insuffisant. En outre, Dingell, Joiner et Hurwitz (1966) et Marshall et McLean (1969) ont montré qu'un tel déséquilibre nutritionnel inhibe en partie l'activité des hydroxylases hépatiques des microsomes du foie.

Nous étudions les effets de chacun des colorants et de la carence protéique sur le métabolisme glucidique en déterminant les activités de la phosphatase et de la déshydrogénase du 6-phosphorylglucose ainsi que l'activité de la déshydrogénase du 6-phosphorylgluconate. Nous mesurons aussi les effets sur le métabolisme des composés exogènes. Nous déterminons l'activité d'une réductase (azoréductase du Bordeaux S) et l'activité d'une oxydase (*N*-déméthylase de l'aminopyrine).

METHODES EXPERIMENTALES

Animaux et traitement. Les animaux sont des rats mâles de souche Wistar CF, specific-pathogen-free. Nous les recevons au sevrage à 30 jours. Nous leur donnons, après 2 jours

d'acclimatation, un régime semi-synthétique qui est soit équilibré (avec 18,18 % en poids de protéines), soit hypoprotéique. La composition du régime équilibré de base a été précisé par ailleurs (Albrecht, Manchon et Lowy, 1972). Le régime hypoprotéique comporte protides (9,05 %), lipides (3,11 %), glucides assimilables (67,02 %), humidité (10,7 %), ballast (5,78 %), matières minérales (4,24 %) et vitamines ajoutées (acides folique et *p*-aminobenzoïque, biotine, ménadione, B_1, B_2, B_6, PP, A, D_3, E, acides gras essentiels et choline, 0,07 %). Les rats témoins reçoivent ces régimes sans autre addition. Pour les animaux traités, nous incorporons à 100 g de régime soit 0,3 g de Bordeaux S, soit 0,06 g de Jaune de Beurre. La durée du traitement est de 1 mois. Les rats traités par le colorant alimentaire ont absorbé 1,3 g de Bordeaux S; ceux qui l'ont été par le cancérogène ont absorbé 0,25 g de Jaune de Beurre.

Après 1 mois, les animaux sont pesés et sacrifiés, les foies sont pesés et un surnageant d'homogénéisat de foie est preparé comme nous l'avons précédemment décrit (Albrecht *et al.* 1973), mais pour cette expérience, les animaux ne sont pas à jeun lors du sacrifice. La teneur en protéines du surnageant est déterminée selon la méthode de Lowry, Rosebrough, Farr et Randall (1951).

Mesure des activités enzymatiques. La mesure des activités de la phosphatase du 6-phosphorylglucose (E.C. 3-1-3-9), de la déshydrogénase du 6-phosphorylglucose (E.C. 1-1-1-49), de la déshydrogénase du 6-phosphorylgluconate (E.C. 1-1-1-43) et de l'azoréductase du Bordeaux S est conduite selon des méthodes que nous avons déjà signalées (Albrecht *et al.* 1973). L'activité de la *N*-déméthylase de l'aminopyrine est déterminée selon la méthode décrite par Gilbert et Golberg (1965). Cependant, contrairement à ces auteurs, nous n'ajoutons pas de déshydrogénase du 6-phosphorylglucose dans le milieu d'incubation. Des expériences préliminaires nous indiquent que l'activité de la *N*-déméthylase est bien plus forte si l'on utilise des quantités relativement importantes de déshydrogénase (5 UI/ml), mais une analyse statistique (étude de la régression linéaire) montre que l'activité endogène de la déshydrogénase du 6-phosphorylglucose ne modifie pas l'activité de l'enzyme étudiée. Nous ne déterminons donc pas l'activité potentielle de la *N*-déméthylase de l'aminopyrine mais une activité plus proche de l'activité physiologique.

RESULTATS

Caractéristiques des animaux

Le colorant alimentaire, le Bordeaux S, ne modifie ni le poids vif des animaux, ni le poids du foie (Tableau 1). Il est sans influence sur la concentration des protéines du surnageant. Les rats traités au colorant cancérigène, le Jaune de Beurre, sont très significativement (13 %) amaigris. Le poids de leur foie est significativement plus important (8 %) que celui des témoins. Le Jaune de Beurre ne semble pas modifier sensiblement la concentration des protéines du surnageant.

Le poids de tous les rats nourris avec un régime hypoprotéique est très significativement plus faible (29 %) que celui des animaux nourris avec un régime équilibré. La réduction des protéines produit toujours une diminution significative du poids des foies (15 %) ainsi que de la concentration des protéines du surnageant (13 %).

Activités enzymatiques

Effets du Bordeaux S. L'administration du colorant alimentaire n'exerce jamais d'influence sur les activités des enzymes que nous étudions (Tableau 2).

Tableau 1. *Poids corporel, poids des foies et concentration des protéines du surnageant de foie des rats traités au Bordeaux S ou Jaune de Beurre et nourris avec un régime équilibré ou hypoprotéique*

Additif (% de régime)	Poids corporel (g)	Poids des foies (g)	Concentration des protéines du surnageant (g/100 g de foie)
	Régime équilibré		
—(Témoins)	208,9 ± 4,53	8,48 ± 0,280	12,43 ± 0,399
Bordeaux S (0,3)	209,7 ± 4,53	8,04 ± 0,280	13,08 ± 0,399
Jaune de Beurre (0,06)	182,8 ± 4,53**	9,15 ± 0,280	12,75 ± 0,399
	Régime hypoprotéique		
—(Témoins)	155,3 ± 4,53**	7,14 ± 0,280	10,92 ± 0,399*
Bordeaux S (0,3)	146,5 ± 4,53**	6,84 ± 0,280*	11,17 ± 0,399*
Jaune de Beurre (0,06)	128,2 ± 4,53**	7,86 ± 0,280	11,09 ± 0,399*

Les valeurs sont les moyennes, écart-type sur la moyenne, calculé de l'écart commun dans les groupes, pour 12 animaux/groupe; celles qui sont marquées avec des astérisques diffèrent significativement de celles des témoins: $*P<0,05$; $**P<0,01$.

Effets du Jaune de Beurre. Le colorant cancérogène abaisse très significativement (24%) l'activité de la phosphatase du 6-phosphorylglucose (Tableau 2). Avec un régime équilibré, les rats traités par le Jaune de Beurre présentent une activité de la déshydrogénase du 6-phosphorylglucose très significativement plus importante que celle des témoins (95%). L'activité de la déshydrogénase du 6-phosphorylgluconate est augmentée très significativement (16%) quelle que soit la teneur en protéines des régimes. Le colorant cancérogène stimule toujours l'azoréductase mesurée avec le Bordeaux S comme substrat (21%). Il abaisse très significativement l'activité de la *N*-déméthylase de l'aminopyrine (42%).

Effets de la réduction des protéines. Chez les animaux témoins ou traités au Bordeaux S, la réduction des protéines alimentaires produit une augmentation très significative (16%) de l'activité de la phosphatase du 6-phosphorylglucose; par contre, chez les rats traités au Jaune de Beurre, la réduction n'a pas d'effet (Tableau 2). De plus, la carence protéique modifie significativement l'action du colorant cancérogène; en effet, l'aminoazoïque abaisse de 24% l'activité de la phosphatase lorsque le régime est équilibré, mais la réduction est de 34% lorsque le régime est hypoprotéique.

L'activité de la déshydrogénase du 6-phosphorylglucose n'est pas modifiée par la réduction des protéines alimentaires chez les rats témoins ou traités au Bordeaux S (Tableau 2). En revanche, chez les animaux traités au Jaune de Beurre, nous observons une diminution très significative (37%). Le régime hypoprotéique modifie l'action du Jaune de Beurre. Nous avons vu que le cancérogène induit la déshydrogénase du 6-phosphorylglucose lorsque le régime est équilibré mais cette induction est empêchée si le régime n'est pas suffisament riche en protéines.

Chez tous les animaux nourris avec un régime hypoprotéique, les activités de la déshydrogénase du 6-phosphorylgluconate et de l'azoréductase (exprimées pour 100 mg de protéines) sont toujours identiques à celles des rats nourris avec un régime équilibré (Tableau 2). En fait, les biosynthèses sont bien réduites par le régime hypoprotéique mais cet effet n'est pas plus sensible pour ces enzymes qu'il ne l'est pour l'ensemble de la masse protéique.

Chez tous les rats nourris avec un régime hypoprotéique, l'activité de la *N*-déméthylase de l'aminopyrine est significativement réduite (18–42%).

Tableau 2. *Activités enzymatiques du foie des rats traités au Bordeaux S ou Jaune de Beurre et nourris avec un régime équilibré ou hypoprotéique*

Additif (% de régime)	Phosphatase du G6P (μmoles P/min/100 mg protéines)	Déshydrogénase du G6P (μmoles NADPH/min/100 mg protéines)	Déshydrogénase du 6PG (μmoles NADPH/min/100 mg protéines)	Azoréductase du Bordeaux S (nmoles de Bordeaux S métabolisées/min/100 mg protéines)	*N*-Déméthylase de l'aminopyrine (nmoles d'aminoantipyrine/min/100 mg protéines)
			Régime équilibré		
—(Témoins)	8,17 ± 0,331	3,94 ± 0,611	10,81 ± 0,724	107,9 ± 7,03	4,70 ± 0,553
Bordeaux S (0,3)	8,27 ± 0,331	3,51 ± 0,611	9,46 ± 0,724	105,9 ± 7,03	4,25 ± 0,553
Jaune de Beurre (0,06)	6,18 ± 0,331**	7,69 ± 0,611**	12,55 ± 0,724	129,1 ± 7,03*	2,74 ± 0,553*
			Régime hypoprotéique		
—(Témoins)	9,98 ± 0,331**	4,81 ± 0,611	9,31 ± 0,724	102,7 ± 7,03	3,60 ± 0,553
Bordeaux S (0,3)	9,11 ± 0,331	3,41 ± 0,611	9,05 ± 0,724	101,3 ± 7,03	3,48 ± 0,553
Jaune de Beurre (0,06)	6,60 ± 0,331**	4,84 ± 0,611	11,45 ± 0,724	141,3 ± 7,03**	1,59 ± 0,553**

G6P = 6-Phosphorylglucose 6PG = 6-Phosphorylgluconate

Les valeurs sont les moyennes, écart-type sur la moyenne, calculé de l'écart commun dans les groupes, pour 12 animaux/groupe; celles qui sont marquées avec des astérisques diffèrent significativement de celles des témoins: $*P < 0,05$; $**P < 0,01$.

DISCUSSION

Nous constatons une nouvelle fois que le Bordeaux S n'a pas d'effet très sensible sur l'organisme du rat en ingestion prolongée. De plus, les animaux traités par le colorant alimentaire ne sont pas plus influencés par la carence protéique que ne le sont les témoins.

Nous confirmons que le Jaune de Beurre produit des modifications du métabolisme cellulaire qui sont en corrélation avec sa toxicité. Il abaisse l'activité de la phosphatase du 6-phosphorylglucose, il diminue le poids vif, il provoque une hépatomégalie et il inhibe la production des enzymes oxydatives microsomales (*N*-déméthylase de l'aminopyrine). En effet, selon Gilbert et Golberg (1965), la conjugaison de ces manifestations caractérise bien l'action d'un hépatotoxique.

La réduction de la teneur en protéines alimentaires entraîne, chez tous les rats, un sensible amaigrissement; il en résulte donc pour les animaux nourris avec un régime hypoprotéique contenant du Jaune de Beurre, une très importante diminution du poids vif puisque l'aminoazoïque, par lui-même, a déjà un tel effet. Nous constatons que la carence protéique augmente l'activité de la phosphatase du 6-phosphorylglucose chez les rats témoins ou traités au Bordeaux S. Ceci est peut-être dû à la plus grande richesse en glucides de ces régimes. Néanmoins, chez les animaux traités par le cancérogène, nous n'observons plus une telle induction; ce fait est à rapprocher de l'observation de Fiala et Fiala (1959) selon laquelle l'augmentation de l'activité de la phosphatase par la cortisone est empêchée dans le foie précancéreux. Le Jaune de Beurre produit donc une nette inhibition de la synthèse de la phosphatase du 6-phosphorylglucose; la carence protéique paraît même accentuer cet effet. Chez les rats témoins ou traités au Bordeaux S, la réduction des protéines alimentaires n'a pas d'action spécifique sur l'activité potentielle de la déshydrogénase du 6-phosphorylglucose. En revanche, elle provoque une inhibition chez les animaux traités au Jaune de Beurre et elle empêche l'induction de l'enzyme due à l'effet propre de ce colorant. La faible teneur en protéines alimentaires ne permet donc pas la synthèse de la déshydro-

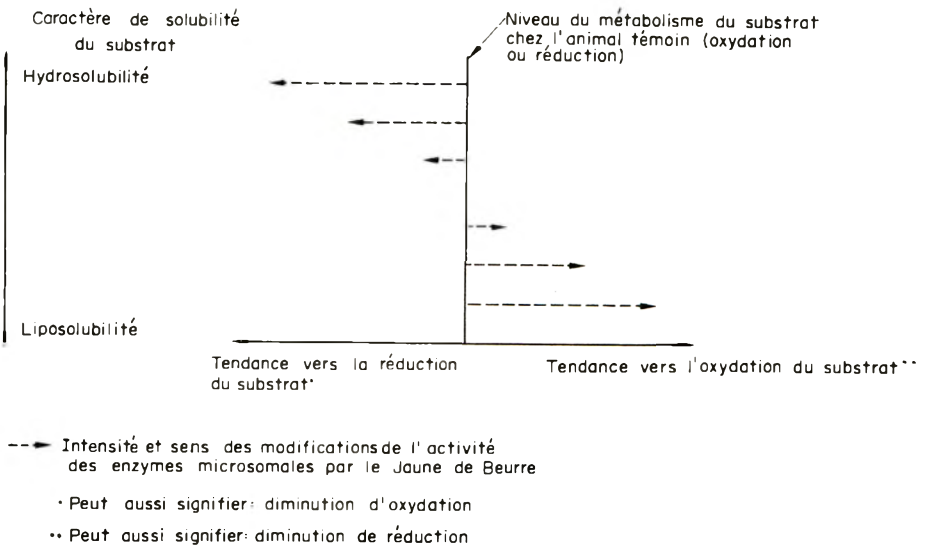


FIG. 1. Représentation hypothétique de l'influence d'une ingestion prolongée du Jaune de Beurre sur l'activité des enzymes microsomales hépatiques.

génase du 6-phosphorylglucose au-delà d'un certain niveau. Enfin, chez tous les rats, l'activité de la *N*-déméthylase de l'aminopyrine est spécifiquement abaissée par la réduction des protéines alimentaires; ceci est en accord avec les observations de Marshall et McLean (1969). Il en résulte pour les animaux nourris avec un régime hypoprotéique contenant du Jaune de Beurre, une très importante diminution de l'activité de l'enzyme puisque le colorant par lui-même a déjà un tel effet.

En définitive, il apparaît que l'administration de Jaune de Beurre modifie très sensiblement le métabolisme microsomal des composés exogènes. Elle abaisse la vitesse d'oxydation d'un composé hydrosoluble (oxydation de l'aminopyrine), elle augmente la réduction d'un composé hydrosoluble (réduction du Bordeaux S) et elle abaisse la réduction d'un composé liposoluble (réduction du Jaune de Beurre, cf. Albrecht *et al.* 1972).

Si le traitement par le Jaune de Beurre augmentait la vitesse d'oxydation d'un composé liposoluble, il serait tentant de bâtir le schéma hypothétique de la Fig. 1, sur laquelle on peut voir que le traitement par le cancérogène orienterait l'activité des enzymes microsomales hépatiques vers la réduction des composés hydrosolubles et vers l'oxydation des liposolubles. Des recherches ultérieures seront nécessaires pour fonder sérieusement cette hypothèse. Néanmoins, si elle se révèle exacte, ce processus présenterait un intérêt fondamental puisqu'il pourrait signifier que le matériel génétique régularise le métabolisme des substances exogènes de manière différente selon la nature de leur solubilité.

REFERENCES

Albrecht, R., Manchon, Ph., Keko-Pinto, C. et Lowy, R. (1973). Evolution, au cours du temps de quelques effets du Bordeaux S et du Jaune de Beurre sur l'organisme du rat. *Fd Cosmet. Toxicol.* **11,** 175.

Albrecht, R., Manchon, Ph. & Lowy, R. (1972). Are there two liver azoreductases? *Fd Cosmet. Toxicol.* **10,** 443.

Dingell, J. V., Joiner, P. D. & Hurwitz, L. (1966). Impairment of hepatic drug metabolism in calcium deficiency. *Biochem. Pharmac.* **15,** 971.

Fiala, S. & Fiala, Anna E. (1959). Intracellular localization of carcinogen and its relationship to the mechanism of carcinogenesis in rat liver. *Br. J. Cancer* **13,** 236.

Gilbert, D. & Golberg, L. (1965). Liver response tests. III. Liver enlargement and stimulation of microsomal processing enzyme activity. *Fd Cosmet. Toxicol.* **3,** 417.

Lowry, O. H., Rosebrough, Nira J., Farr, A. L. & Randall, Rose J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265.

Marshall, W. J. & McLean, A. E. M. (1969). The effect of oral phenobarbitone on hepatic microsomal cytochrome P-450 and demethylation activity in rats fed normal and low protein diets. *Biochem. Pharmac.* **18,** 153.

Influence of the protein level of the diet on some effects of amaranth and butter yellow in the rat

Abstract—The effects of a reduction in dietary protein have been studied in control rats and in those treated either with a food additive (amaranth) or a hepatocarcinogen (butter yellow). The animals treated with amaranth did not differ from the controls in their response to the protein deficiency. On the other hand, the reduction in dietary protein appeared to accentuate the depression of glucose 6-phosphatase activity caused by butter yellow, and prevented induction of glucose-6-phosphate dehydrogenase by the hepatocarcinogen. In all the animals, the low-protein diet specifically lowered the activity of aminopyrine *N*-demethylase. It is possible that *in vitro* microsomal metabolism of foreign compounds differs according to the type of solubility exhibited by the substrate.

Einfluss der Proteinkonzentration des Futters auf einige Wirkungen von Amaranth und Buttergelb bei der Ratte

Zusammenfassung—Der Einfluss einer Verminderung des Proteinanteils im Futter wurde an Kontrollratten und bei solchen Tieren untersucht, die entweder einen Lebensmittelzusatz (Amaranth) oder ein Lebercarcinogen (Buttergelb) erhalten hatten. Die Tiere, die Amaranth erhielten, unterschieden sich in ihrer Reaktion auf den Proteinmangel nicht von den Kontrolltieren. Andererseits schien die Proteinverminderung im Futter die von Buttergelb verursachte Depression der Glucose-6-phosphataseaktivität zu betonen und verhinderte die Induktion von Glucose-6-phosphatdehydrogenase durch das Lebercarcinogen. Bei allen Tieren verminderte das proteinarme Futter speziell die Aktivität von Aminopyrin-*N*-demethylase. Es ist möglich, dass sich der mikrosomale *in-vitro*-Metabolismus von Fremdsubstanzen entsprechend der Art von Löslichkeit, die das Substrat zeigt, unterscheidet.

Fd Cosmet. Toxicol. Vol. 11, pp. 391–402. Pergamon Press 1973. Printed in Great Britain

The Metabolism of Saccharin in Laboratory Animals*

J. L. BYARD and L. GOLBERG

Institute of Experimental Pathology and Toxicology, Albany Medical College of Union University, Albany, New York 12208, USA

(*Received 1 November 1972*)

Abstract—When rats and monkeys of both sexes received a single oral dose of 40 mg sodium [^{14}C]saccharin/kg, more than 90% was recovered in the urine collected for 96 hr after dosing. Thin-layer chromatography of the whole urine indicated that the only significant radioactivity on the developed chromatograph had the same R_F as saccharin; this radioactive fraction recrystallized with pure unlabelled saccharin. Hydrolysis products of saccharin were not detected. Artefactual 'metabolites' of saccharin were observed if radioactive contaminants were not accounted for, if control urine was not compared with test urine and if saccharin was extracted from urine under acidic conditions.

During the first 4–8·75-hr collection period, no more than 0·3% of an oral dose of saccharin was excreted in rat bile, while up to 11·7% passed into the bladder. The ^{14}C-fraction in the bile had the same R_F as saccharin. Induction of mixed function oxidase activity in rat liver by treatment with phenobarbitone had no influence on saccharin metabolism. Daily oral doses of 20, 100 or 500 mg sodium saccharin/kg, given to monkeys for over 2 yr, had no effect on the metabolism of a single oral dose of [^{14}C]saccharin. We conclude that, in the species studied and within the limits of our analytical methods, saccharin is not metabolized.

INTRODUCTION

An important aspect of the safety evaluation of a food additive is the detection, identification and toxicological study of its metabolites. In the case of saccharin, investigations with non-isotopic material have indicated that there is no metabolic transformation of the molecule (Mathews & McGuigan, 1905; Uglow, 1924), but recent studies, using radioactive saccharin, have revealed the presence of low levels of metabolites in urine. These metabolites have been tentatively identified as *o*-sulphamoylbenzoic acid and *o*-sulphobenzoic acid, the hydrolysis products of saccharin (Kennedy, Fancher, Calandra & Keller, 1972; Pitkin, Andersen, Reynolds & Filer, 1971). The urine collected over certain intervals contained more than 60% of the radioactivity in the form of metabolites (Pitkin *et al.* 1971). The experiments reported herein were designed to detect and measure metabolites of saccharin in the urine of rats and monkeys.

EXPERIMENTAL

Standards and doses. Saccharin, as the free acid, was obtained from Matheson, Coleman and Bell (Norwood, Ohio) and *o*-sulphamoylbenzoic acid and *o*-sulphobenzoic acid were provided by Monsanto Co. (St. Louis, Mo.). All three chemicals were checked for purity by

*Presented in part at the Eleventh Annual Meeting of the Society of Toxicology, Williamsburg, Virginia, 5–9 March 1972.

thin-layer chromatography, titration, melting point and infra-red spectroscopy. They were at least 99% pure. Radioactive saccharin was obtained from Mallinckrodt Chemical Works (St. Louis, Mo.) as [^{14}C]saccharin uniformly labelled in the benzene ring. In preliminary experiments, the material administered was a mixture of unlabelled saccharin and [^{14}C]-saccharin, neutralized with NaOH. To prepare material for a purified dose, a ^{14}C-contaminant in the [^{14}C]saccharin was removed by preparative thin-layer chromatography on layers of silica gel 2 mm thick (Brinkman Instruments, Inc., Westbury, N.Y.), developed with solvent A (2-propanol–water–dimethylformamide, 92:6:2, by vol.). The centre of the saccharin peak was scraped into a sintered glass filter and eluted with methanol. The methanol was evaported and the residue was taken up in water, neutralized with NaOH and diluted with unlabelled sodium saccharin. The specific activity of the dose containing the purified material was 0·716 μC/mg saccharin for rats and 3·57 μC/mg saccharin for monkeys. In all experiments the animals received 40 mg [^{14}C]saccharin/kg, as the sodium salt, by stomach tube.

Urine collection. Two rhesus monkeys (*Macaca mulatta*) of each sex (body weight 3·5–5·7 kg), and four male (228–265 g) and two female (321 and 344 g) Sprague–Dawley rats were dosed with purified [^{14}C]saccharin, and urine was collected in the presence of chloroform for 24-hr intervals up to 96 hr. Faeces were kept separate from urine by using a curled-in dripspout on the rat metabolism cages, by using a large funnel covered with wire mesh for the female monkeys, and by tying a cup into the crotch of the male monkeys. A tube ran from the cup into a receiving vessel containing a small volume of chloroform. All animals received water *ad lib.* during the collection period; the rats were fasted, but the monkeys received Purina Monkey Chow. A dose of [^{14}C]saccharin comparable to 40 mg/kg of the control animal was added to the collection vessels of undosed animals, and urine was collected under the same conditions as those used for the test animals.

Chromatography of urine and faeces. Analytical or preparative silica-gel thin-layer plates were obtained from Brinkman Instruments, Inc., streaked with filtered urine and developed 15 cm with solvent A. The standards were visualized by spraying the dried plates with 0·2% bromocresol green in methanol; they appeared as yellow spots against a blue-green background. The plates were divided into 30 equal sections from origin to solvent front, each section was scraped into 10 ml of scintillation fluid (Patterson & Greene, 1965), 1 ml water was added and the vials were assayed for ^{14}C in a Packard Tricarb Liquid Scintillation Spectrometer. The radioactivity in each section was plotted as the percentage of the total radioactivity on the chromatograph against the sections along the chromatograph from origin to solvent front. Urinary radioactivity that chromatographed with the same R_F as *o*-sulphamoylbenzoic acid was purified by chromatography on XAD-2 resin (Rohm and Hass Co., Philadelphia, Pa.), on silica-gel layers developed with solvent A and on cellulose layers (Brinkman Instruments, Inc.) developed with solvent C (acetone–acetonitrile–water–ammonia, 32:52:13:3, by vol.).

A comparison was made between the results of direct chromatography of urine and those of preliminary extraction with acid chloroform–ethyl acetate (Kennedy & Fancher, 1970) followed by thin-layer chromatography. Rat and monkey urine was collected for up to 96 hr after dosing, acidified to pH 2 with HCl, extracted three times with 2 vols chloroform, and three times with 2 vols ethyl acetate. The chloroform and ethyl acetate fractions were dried at room temperature under a stream of air. The residues were redissolved in acetone and streaked on thin-layer plates for chromatography. The figures represent the results of the combined chloroform and ethyl acetate extracts.

Rat faeces were collected for 48 hr after dosing, dried, ground into a powder and extracted three times with methanol on a sintered glass filter. The methanol extracts were combined, streaked on thin layers of silica gel and developed with solvent A.

Recrystallization of saccharin and o-*sulphamoylbenzoic acid.* The radioactive urinary fractions that chromatographed with the same R_F as saccharin and *o*-sulphamoylbenzoic acid were separated by preparative thin-layer chromatography as described above. The centre of each of the two peaks was scraped from the plate and washed repeatedly with methanol. The methanol extract of the fraction with the same R_F as saccharin was added to 200 mg pure saccharin, and the corresponding extract of material with the same R_F as *o*-sulphamoylbenzoic acid was added to 200 mg pure *o*-sulphamoylbenzoic acid. All acids were converted to their ammonium salts and the excess ammonium hydroxide was evaporated at 65°C. The saccharin was then recrystallized five times from a mixture of methanol and 2-propanol (50:50, v/v). Each batch of crystals was obtained by evaporating the solvent at 65°C until crystallization set in. At this point, the samples were cooled in ice for 2–3 hr to crystallize. The crystals were filtered, washed twice with a minimum volume of isopropyl ether, dried to constant weight at 65°C and redissolved in 4 ml of the 2-propanol–methanol mixture. A small aliquot was taken for radioactivity determination and the recrystallization was continued. The same procedure was used for *o*-sulphamoylbenzoic acid, except that the solvent used was methanol. Specific activity was expressed as disintegrations/min/mg of crystalline material.

Collection of bile. Male and female rats were given [^{14}C]saccharin by stomach tube and were anaesthetized with ether. Their bile ducts were cannulated and bile was collected for periods lasting from 4 to 8·75 hr. After the collection period, the animals were killed with ether and the urine was removed from the bladder. The urine was streaked directly on analytical silica-gel layers and developed with solvent A. The bile was diluted to 5 ml with HCl and water to give a final pH of 2. Repeated chloroform extractions were made until more than 90% of the ^{14}C had been extracted. The chloroform phase was streaked directly on preparative silica-gel layers and developed with solvent A.

Phenobarbitone treatment. Female rats weighing 150 g were given 50 mg phenobarbitone/kg by stomach tube daily for 3 days. On day 4, controls and the treated rats received a single oral dose of [^{14}C]saccharin. Urine was collected for 24 hr, filtered, streaked directly on analytical silica-gel layers and developed with solvent A. The rats were sacrificed immediately after collection of the urine, and the livers were removed and homogenized in a medium of 0·25 M-sucrose in 0·01 M-tris chloride, pH 7·4. The homogenate was centrifuged for 10 min at 10,000 **g** and the supernatant was assayed fluorimetrically for biphenyl-4-hydroxylase activity (Creaven, Parke & Williams, 1965).

Metabolism of saccharin in monkeys given saccharin for over 2 yr. Male rhesus monkeys were given saccharin, as the sodium salt, in a dose of 0, 20, 100 or 500 mg/kg by stomach tube once a day on 6 days/wk for 26 months. The normal dose was replaced with 40 mg [^{14}C]saccharin/kg and urine was collected for 24 hr. The monkeys received water *ad lib.* and were hand-fed Purina Monkey Chow. The urine was filtered, streaked directly on Gelman fibre-glass sheets impregnated with silica gel (Gelman Instrument Co., Ann Arbor, Mich.), and developed with solvent E (2-propanol–water–dimethylformamide–ammonia, 89:6:4:1, by vol.).

RESULTS

Chromatography of pure saccharin, o-sulphamoylbenzoic acid and o-sulphobenzoic acid. When silica-gel thin-layer plates were streaked with saccharin, *o*-sulphamoylbenzoic acid and *o*-sulphobenzoic acid, developed with solvent A and sprayed with bromocresol green, the R_F values were 0·61, 0·41 and 0·06 respectively. When the plates were developed with solvent E, the corresponding values were 0·67, 0·51 and 0·07.

Breakdown product of the ^{14}C-contaminant in the [^{14}C]saccharin. [^{14}C]Saccharin was stable in control urine for up to 96 hr at room temperature. About 95% of the ^{14}C in the urine collected for 24 hr after male rats or monkeys had been dosed with [^{14}C]saccharin chromatograped with the same R_F as saccharin. About 2–3% chromatographed with the same R_F as *o*-sulphamoylbenzoic acid. When the centre sections of this radioactive component were eluted and mixed with pure *o*-sulphamoylbenzoic acid, the ^{14}C did not crystallize with the added compound. In one experiment, using ^{14}C from rat urine, 72% of the *o*-sulphamoylbenzoic acid was precipitated while 91% of the ^{14}C remained in solution. Apparently the radioactive component with the same R_F as *o*-sulphamoylbenzoic acid was not *o*-sulphamoylbenzoic acid. Purification of this radioactive component from monkey urine was continued until the weight of the purified fraction was less than 0·2 mg. At this point, the specific activity was greater than 10^7 disintegrations/min/mg of material, which was 50 times the specific activity of the [^{14}C]saccharin from the same urine. Therefore this radioactive component appeared to be a metabolite or breakdown product of a ^{14}C-contaminant in the [^{14}C]saccharin and was not present in the unlabelled saccharin which was used to dilute the [^{14}C]saccharin in preparing the dose. To test this possibility, the [^{14}C]saccharin was chromatographed on a preparative layer of silica gel with solvent A, and the centre of the saccharin peak was eluted from the developed chromatograph. From the eluted [^{14}C]saccharin, a dose of saccharin was prepared in the usual manner and given to rats and monkeys. Purification of the [^{14}C]saccharin removed a trailing shoulder on the saccharin peak (Fig. 1). The removal of this shoulder caused a proportionate decrease in the radioactive component with the same R_F as *o*-sulphamoylbenzoic acid in the 0–24-hr monkey urine (Fig. 1). A similar result was obtained with rats. Purification of the ^{14}C-contaminant by preparative thin-layer chromatography, followed by elution and rechromatography in the same system, demonstrated that the ^{14}C-contaminant broke down spontaneously to the radioactive component with the same R_F as *o*-sulphamoylbenzoic acid (Fig. 2). In view of these findings, purified [^{14}C]sacchrian was used to determine the presence of metabolites in urine collected from rats and monkeys for up to 96 hr after dosing.

Saccharin metabolism in rats and monkeys up to 96 hr after dosing. Most of the ^{14}C administered was recovered in the urine during the first 24 hr (Figs 3 & 4). Direct chromatography of the urine revealed only saccharin; that is, hydrolytic products were not detected. There was not enough ^{14}C in the 48–72- and 72–96-hr collections of monkey urine to carry out direct chromatography of these specimens. Direct chromatography of the 0–24-hr collection of urine from mice, golden hamsters, guinea-pigs and dogs after a single oral dose of 40 mg [^{14}C]saccharin/kg indicated that also in these species saccharin was the only significant ^{14}C-labelled compound excreted.

Only 3–4% of the dose was excreted in the 48-hr collection of faeces from rats. About 90% of the ^{14}C in the faeces was extracted by methanol and all of this ^{14}C chromatographed as saccharin.

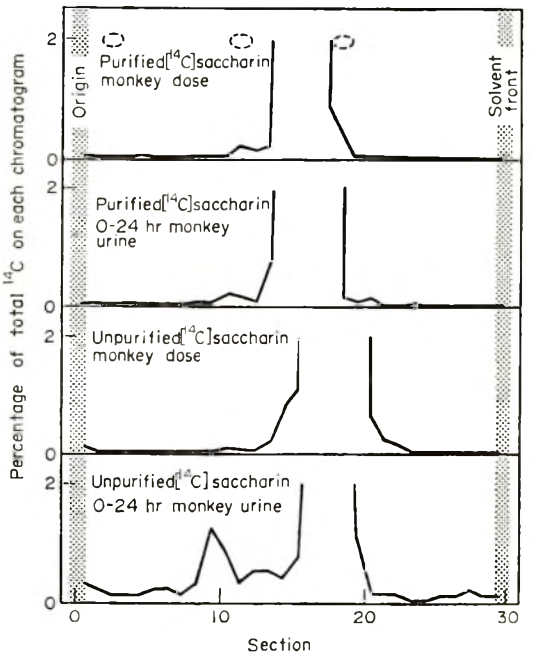


FIG. 1. Chromatography of the ^{14}C-contaminant and its breakdown product in purified and unpurified [^{14}C]saccharin. The 0–24 hr urine was collected from monkeys given an oral dose of 40 mg [^{14}C]saccharin/kg, as the sodium salt. The results are representative data from one of two monkeys. The broken circles represent the average positions of *o*-sulphobenzoic acid (section 2), *o*-sulphamoylbenzoic acid (section 12) and saccharin (section 18). The chromatographs were developed with solvent A.

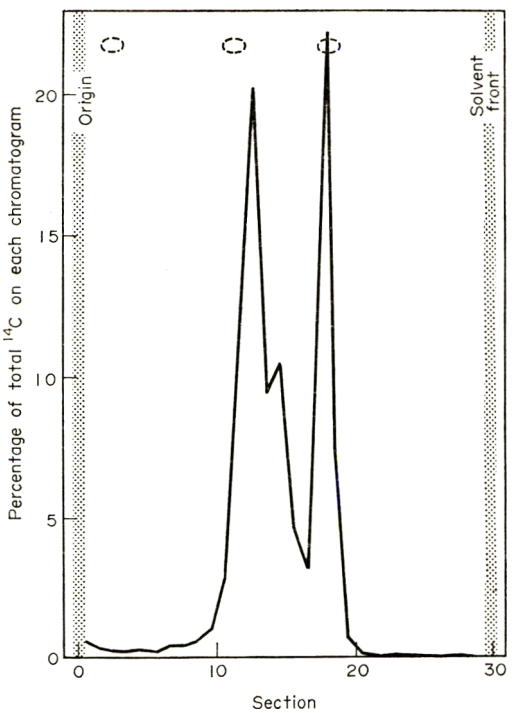


FIG. 2. Chromatography of the purified ^{14}C-contaminant. The peaks represent the breakdown product of the ^{14}C-contaminant (section 12), the contaminant itself (section 15) and saccharin (section 18). The broken circles have the same meaning as in Fig. 1. The chromatographs were developed with solvent A.

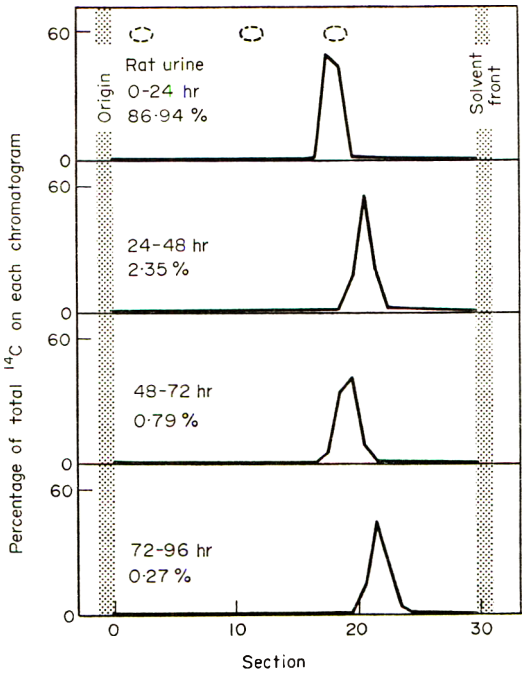


FIG. 3. Direct chromatography of ^{14}C in urine collected for up to 96 hr from rats given an oral dose of 40 mg [^{14}C]saccharin/kg, as the sodium salt. The percentages represent that part of the dose recovered in the urine during each collection period. The results are representative data from one of four rats. The broken circles have the same meaning as in Fig. 1. The chromatographs were developed with solvent A.

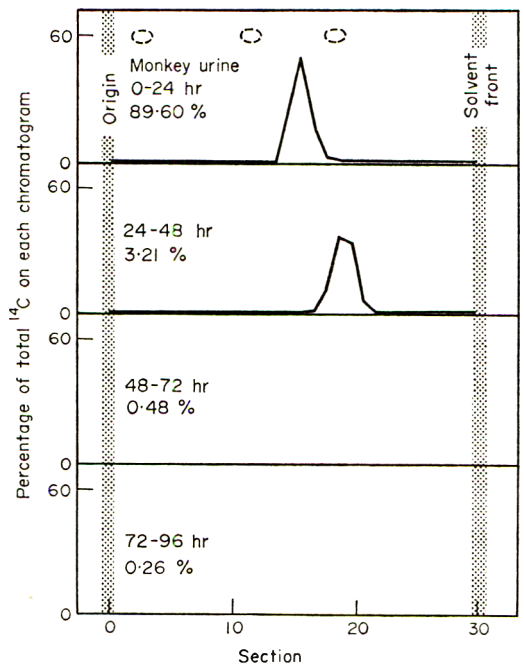


FIG. 4. Direct chromatography of ^{14}C in urine collected for up to 48 hr from monkeys given an oral dose of 40 mg [^{14}C]saccharin/kg, as the sodium salt. The results are representative data from one of two monkeys. The percentages represent that part of the dose recovered in the urine during each collection period. The broken circles have the same meaning as in Fig. 1. The chromatographs were developed with solvent A.

The absence of any detectable metabolism of saccharin is in conflict with other isotopic studies where as little as a trace (Kennedy *et al.* 1972) to as much as 60% (Pitkin *et al.* 1971) of the ^{14}C from some collection periods was found to be in the form of the hydrolytic products of saccharin. To clarify these differences, the results of direct chromatography of urine were compared with those produced by acidification to pH 2 followed by extraction with chloroform and ethyl acetate. Since the extraction method concentrates the ^{14}C away from the urinary solids, specimens of urine from all of the collection periods could be chromatographed (Figs 5 & 6). Although neither of the hydrolytic products of saccharin was detected, peaks with an R_F greater than that of saccharin were observed in the 24–48-, 48–72- and 72–96-hr urine collections. No such peak was observed when the urine was chromatographed directly (Figs 3 & 4). These results indicate that artefactual peaks may result when the extraction method is used prior to chromatography.

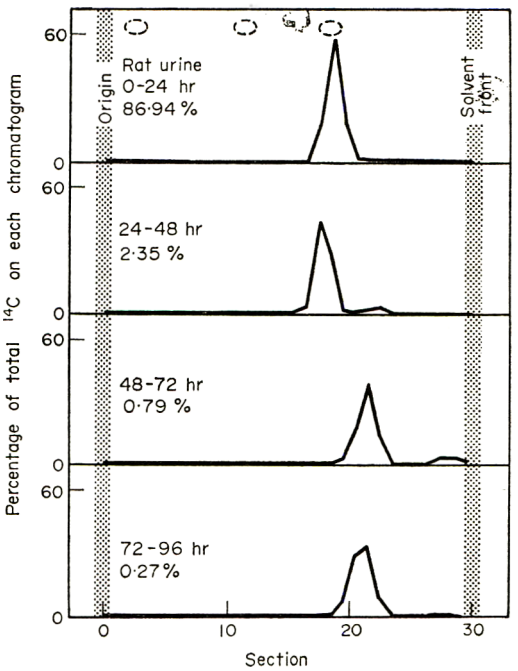


FIG. 5. Chromatography of ^{14}C extracted from rat urine. The urine was collected for up to 96 hr from rats given an oral dose of 40 mg [^{14}C]saccharin/kg, as the sodium salt. The results are respresentative data from one of four rats. The percentages represent that part of the dose recovered in the urine during each collection period. The broken circles have the same meaning as in Fig. 1. The chromatographs were developed with solvent A.

Purity of the saccharin peak. The possibility remained that a metabolite might not have been detected if it chromatographed with the same R_F as saccharin. To verify the identity of the saccharin peak, all of the ^{14}C in the peak from rat urine was eluted from silica gel and recrystallized with pure saccharin. After repeated recrystallization, there was no change in specific activity (Fig. 7). This result was observed in two separate experiments with two different collections of rat urine. In fact, the ^{14}C in the rat and monkey urine chromatographed identically with saccharin when analysed by gas–liquid chromatography, by high-pressure liquid–liquid chromatography and by three thin-layer chromatography systems.

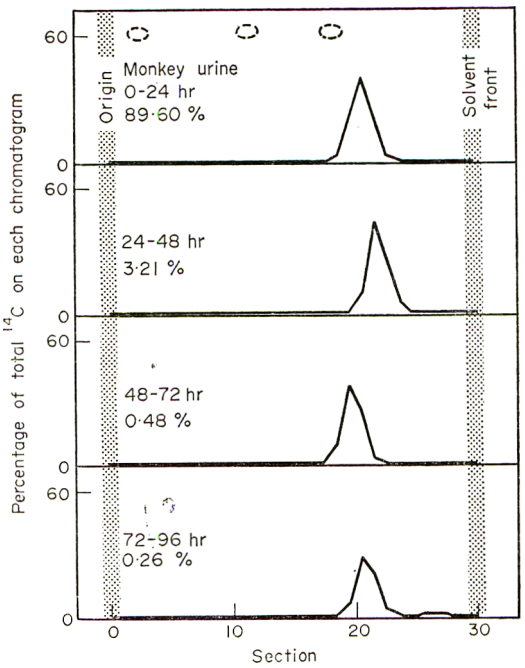


FIG. 6. Chromatography of ^{14}C extracted from monkey urine. The urine was collected from monkeys given an oral dose of 40 mg [^{14}C]saccharin/kg, as the sodium salt. The results are representative data from one of two monkeys. The percentages represent that part of the dose recovered in the urine during each collection period. The broken circles have the same meaning as in Fig. 1. The chromatographs were developed with solvent A.

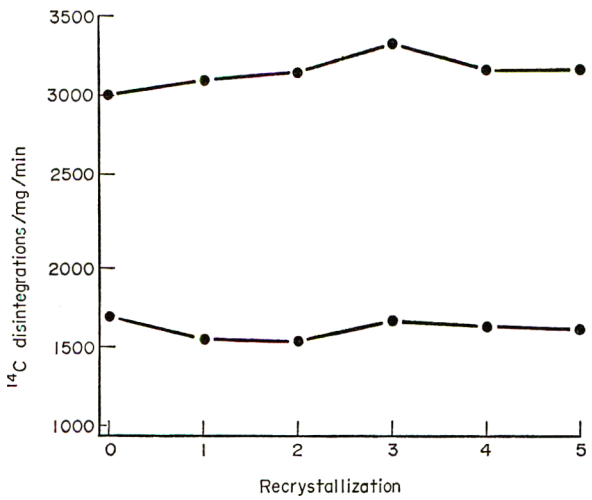


FIG. 7. Recrystallization of the [^{14}C]saccharin peak from rat urine with pure saccharin. The two lines represent separate experiments with the 0–24 hr urine from individual rats given an oral dose of 40 mg [^{14}C]saccharin/kg. Saccharin was converted to the ammonium salt and recrystallized from a mixture of equal volumes of 2-propanol and methanol.

Excretion of saccharin in rat bile. Table 1 summarizes the results of five experiments involving cannulation of the bile duct in rats dosed with [^{14}C]saccharin. While 0·05–0·30% of the dose was excreted in the bile, 2·2–11·7% was excreted in the urine. Thus bile was a minor route of saccharin excretion. Chromatography of the ^{14}C extracted from the bile of rats receiving the purified saccharin showed that more than 97% was saccharin.

Table 1. *Excretion of saccharin in rat bile*

Rats used		[^{14}C]saccharin administered	Collection period (hr)	Proportion of dose (%)	
Sex	Weight (g)			In bile	In bladder urine
Male	270	Unpurified	6·0	0·05	7·26
	330	Unpurified	8·75	0·10	11·66
Female	220	Unpurified	7·75	0·29	7·30
	220	Purified	6·75	0·30	4·57
	210	Purified	4·0	0·05	2·18

All rats received an oral dose of 40 mg [^{14}C]saccharin/kg, as the sodium salt.

Saccharin metabolism in rats treated with phenobarbitone. Although hydroxylation of biphenyl in the 4-position was stimulated by 140%, there was no change in the chromatographic distribution of ^{14}C in the 24-hr urine of rats dosed with [^{14}C]saccharin (Table 2). The only ^{14}C peak accounting for more than 0·2% of the ^{14}C in the urine was saccharin.

Table 2. *Saccharin metabolism in rats treated with phenobarbitone prior to administration of a single dose of [^{14}C]saccharin*

Group	Biphenyl-4-hydroxylase activity* (μmoles 4-hydroxybiphenyl/g fresh liver/hr	Proportion of ^{14}C dose excreted in 0–24 hr urine (%)	Proportion of ^{14}C in urine as saccharin† (%)
Control	0·78 ± 0·07	91 ± 3	99·3 ± 0·1
Phenobarbitone-treated	1·86 ± 0·18	96 ± 3	99·4 ± 0·1

Values are means for groups of three animals ± SD.
*Measured in the 100,000 g/min supernantant by the method of Creaven *et al.* (1965). Average of three animals ± SD.
†The only ^{14}C-peak greater than 0·2% of the ^{14}C in the urine was saccharin. The chromatographs were developed with solvent A.

Saccharin metabolism in monkeys fed saccharin for over 2 yr. Male rhesus monkeys given 20, 100 or 500 mg saccharin/kg, as the sodium salt, daily for 6 days/wk for 26 months did not metabolize a single dose of [^{14}C]saccharin differently from monkeys not previously exposed to saccharin (Table 3). The only ^{14}C peak accounting for more than 0·2% of the ^{14}C in the urine was saccharin.

Table 3. *Saccharin metabolism in monkeys given saccharin orally for over 2 yr*

Daily oral dose of saccharin (mg/kg)	Urinary excretion of single dose of [^{14}C]saccharin	
	Proportion of dose excreted in 0–24 hr urine (%)	Proportion of ^{14}C in urine as saccharin* (%)
0	88	98·8
20	81	99·4
100	84	99·7
500	82	98·7

*The mean value for 2 monkeys is reported at each dose level. The only ^{14}C peak accounting for more than 0·2% of the ^{14}C in the urine was saccharin. The chromatographs were developed with solvent E.

DISCUSSION

Since 1905, it has been known that most of an oral dose of saccharin is excreted in the first 24-hr collection of urine as unchanged saccharin (Mathews & McGuigan, 1905). Recent isotopic experiments (Kennedy *et al.* 1972; Pitkin *et al.* 1971) confirm the earlier findings, as do the results presented in this paper. However, Kennedy *et al.* (1972) and Pitkin *et al.* (1971) found that saccharin was metabolized to *o*-sulphamoylbenzoic acid and *o*-sulphobenzoic acid in rats and monkeys respectively. Although this metabolism did not exceed 1% of the dose in either species, as much as 60% of the ^{14}C in the 48–72-hr urine collection from monkeys was found by Pitkin *et al.* (1971) to be in the form of metabolites. In contrast to these isotopic experiments, the results reported here indicate that there is no metabolism of saccharin in rats or monkeys. The same conclusion has recently been reached for rats and guinea-pigs (Minegishi, Asahina & Yamaha, 1972).

There are several possible explanations for the detection of *o*-sulphamoylbenzoic acid and *o*-sulphobenzoic acid in the urine. If the ^{14}C-contaminant, reported in this paper, was present in the saccharin used in these isotopic experiments, and if the contaminant generated the ^{14}C-breakdown product that chromatographed with *o*-sulphamoylbenzoic acid, then the ^{14}C-contaminant could account for the results reported. The ^{14}C-contaminant chromatographed as a shoulder on the saccharin peak and may have been missed by these workers. One of these reports (Pitkin *et al.* 1971) did not indicate the purity of the [^{14}C]saccharin used in the preparation of the administered dose.

The acid extraction procedure led to artefactual peaks, but these peaks did not chromatograph with the hydrolysis products of saccharin, so the method *per se* did not lead to the reported metabolites. Since an acid environment will hydrolyse saccharin to *o*-sulphamoylbenzoic acid and *o*-sulphobenzoic acid, storage of the urine under acid conditions would lead to the artefactual production of the hydrolysis products of saccharin and provide a possible explanation for the recorded observations.

The delayed excretion of trace amounts of the hydrolysis products, or contaminants that chromatograph with the hydrolysis products, might also account for the high level of 'metabolites' that has been reported in the 48–72-hr collection period. A contaminant at 0·1% of the dose, which may not be detected, would constitute a much greater proportion of the urinary ^{14}C if it were excreted during the later collection periods when less than 1% of the [^{14}C]saccharin was excreted. By further chemical or chromatographic analysis of their radioactive fractions, the workers cited might clarify the differences between their observations and those published in this report.

In summary, artefactual peaks were generated if impurities were not removed from radioactive saccharin or if the urine was extracted at acid pH prior to chromatography. Direct chromatography demonstrated that in the species studied and within the limits of our analytical methods, saccharin was not metabolized. If saccharin is not metabolized in man, a situation which would be predicted from our animal studies, then only saccharin itself needs to be evaluated for safety. This conclusion can only be reached with certainty by studying the metabolism of saccharin in man. Such investigations are in progress and a preliminary report of some of these studies is given in the following paper (McChesney & Golberg, 1973).

Acknowledgements—The authors acknowledge the capable technical assistance of Hai-Chow Kao, S. MacCarthy and R. Roth. This work was supported by the US Food and Drug Administration (Contract 69-7), by the Canadian Food and Drug Directorate, by Research Grant 2 PO1-ES00226-06 from the National Institute of Environmental Health Sciences, NIH, and by National Institutes of Health Training Grant 2 TO1-ES00103-06.

REFERENCES

Creaven, P. J., Parke, D. V. & Williams, R. T. (1965). A fluorimetric study of the hydroxylation of biphenyl *in vitro* by liver preparations of various species. *Biochem. J.* **96,** 879.

Kennedy, G. R. & Fancher, O. E. (1970). Identification and Quantitation of Saccharin and Two Metabolites in Biological Fluids. IBT No. E8218. Unpublished report on file in the office of the Food Protection Committee, National Academy of Sciences.

Kennedy, G., Fancher, O. E., Calandra, J. C. & Keller, R. E. (1972). Metabolic fate of saccharin in the albino rat. *Fd Cosmet. Toxicol.* **10,** 143.

McChesney, E. W. & Golberg, L. (1973). The excretion and metabolism of saccharin in man. I. Methods of investigation and preliminary results. *Fd Cosmet. Toxicol.* **11,** 403.

Mathews, S. A. & McGuigan, H. (1905). The influence of saccharin on the digestive enzymes. *J. Am. med. Ass.* **45,** 844.

Minegishi, K.-I., Asahina, M. & Yamaha, T. (1972). The metabolism of saccharin and the related compounds in rats and guinea pigs. *Chem. pharm. Bull., Tokyo* **20,** 1351.

Patterson, M. S. & Greene, R. C. (1965). Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. *Analyt. Chem.* **37,** 854.

Pitkin, R. M., Andersen, D. W., Reynolds, W. Ann & Filer, L. J., Jr. (1971). Saccharin metabolism in *Macaca mulatta*. *Proc. Soc. exp. Biol. Med.* **137,** 803.

Uglow, W. A. (1924). Über die Wirkung des Saccharins auf Bakterien, Plankton und Verdauungsfermente. *Arch. Hyg. Bakt.* **92,** 331.

Le métabolisme de la saccharine chez les animaux de laboratoire

Résumé—Des rats et des singes des deux sexes ayant reçu une dose orale unique de 40 mg/kg de saccharine sodée marquée au ^{14}C, plus de 90% de celle-ci ont été récupérés dans l'urine recueillie 96 h après l'administration. La chromatographie en couche mince de l'urine totale a indiqué que la seule radioactivité significative relevée après développement du chromatogramme présentait le même R_F que la saccharine; cette fraction radioactive recristallisait avec la saccharine pure non marquée. On n'a pas décelé de produits d'hydrolyse de la saccharine. Des "artefacts métaboliques" de la saccharine ont été observés dans la mesure où l'on n'avait pas tenu compte des polluants radioactifs, où l'on n'avait pas comparé l'urine témoin avec l'urine recueillie pendant l'essai et où l'on avait extrait l'urine dans des conditions acides.

Pendant les 4 à 8,75 premières heures de récupération, pas plus de 0,3% de la dose orale de saccharine n'ont été excrétés dans la bile chez les rats, tandis que jusqu'à 11,7% passaient dans la vessie. La fraction au ^{14}C de la bile avait le même R_F que la saccharine. L'induction chez le rat, par un traitement au phénobarbital, d'une activité oxydasique hépatique à fonction mixte n'a pas influé sur le métabolisme de la saccharine. Des doses orales de 20, 100 ou 500 mg de saccharine sodée par kg et par jour administrées pendant plus de 2 ans à des singes sont restées sans effet sur le métabolisme d'une dose orale unique de saccharine marquée au ^{14}C. Nous concluons que, chez les animaux utilisés pour l'essai et dans les limites de nos méthodes d'analyse, la saccharine n'est pas métabolisée.

Der Stoffwechsel von Saccharin in Laboratoriumstieren

Zusammenfassung—Nachdem Ratten und Affen beider Geschlechter orale Einzeldosen von 40 mg Natrium[^{14}C]saccharin/kg erhalten hatten, wurden über 90% davon in dem 96 Stunden lang nach der Verabreichung ausgeschiedenen und gesammelten Urin wiedergewonnen. Die Dünnschichtchromatographie des gesamten Urins zeigte, dass die einzige signifikante Radioaktivität auf dem entwickelten Chromatogramm den gleichen R_F besass wie Saccharin; diese radioaktive Fraktion kristallisierte mit reinem unmarkiertem Saccharin um. Hydrolyseprodukte von Saccharin wurden nicht beobachtet. Künstliche "Stoffwechselprodukte" von Saccharin wurden beobachtet, wenn radioaktive Verunreinigungen rechnerisch nicht zu erfassen waren, wenn Kontrollurin nicht mit Testurin verglichen wurde und wenn Saccharin aus Urin unter sauren Bedingungen extrahiert wurde.

Während der ersten Sammlungsperiode von der 4. bis zur 8,75ten Stunde wurden nicht mehr als 0,3% einer oralen Dosis von Saccharin mit dem Gallensaft der Ratte ausgeschieden, während bis 11,7% in der Harnblase auftraten. Die ^{14}C-Fraktion im Gallensaft hatte den gleichen R_F wie Saccharin. Die Induktion der Aktivität mischfunktioneller Oxydasen in der Rattenleber durch Verabreichung von Phenobarbitone hatte keinen Einfluss auf den Saccharinstoffwechsel. Tägliche orale Dosen von 20, 100 oder 500 mg Natriumsaccharin/kg, die über 2 Jahre lang an Affen verabreicht wurden, hatten keinen Einfluss auf den Stoffwechsel einer einzelnen oralen Dosis von [^{14}C]Saccharin. Wir schliessen daraus, dass Saccharin bei den untersuchten Tierarten und innerhalb der Grenzen unserer analytischen Methode nicht metabolisiert wird.

Fd Cosmet. Toxicol. Vol. 11, pp. 403–414. Pergamon Press 1973. Printed in Great Britain

The Excretion and Metabolism of Saccharin in Man. I. Methods of Investigation and Preliminary Results*†

E. W. McCHESNEY and L. GOLBERG

Institute of Experimental Pathology and Toxicology, Albany Medical College, Albany, New York 12208, USA

(*Received 22 December 1972*)

Abstract—Methods for the determination of saccharin and its most probable breakdown product (*o*-sulphamoylbenzoic acid; *o*-SAMB) in urine have been developed and applied to a study of the metabolism of saccharin in man. When administered in 1 g doses to three subjects, saccharin appeared to be excreted quantitatively in the urine in unchanged form by two of the subjects within 48 hr. However, in a subsequent and more detailed experiment in six subjects, involving the administration of a dose in the 0·5–1 g range, it was found that none excreted the dose quantitatively as saccharin within 72 hr. Alkaline hydrolysis of the urine samples, using conditions known to convert saccharin quantitatively to *o*-SAMB, increased the overall recoveries to 91–99% of the dose. It was not definitely established what form of saccharin was present to account for the increased recovery on alkaline hydrolysis. Hydrolysis of saccharin to *o*-SAMB *in vivo*, followed by conjugation with glycine was one possibility, but it was also considered possible that part of the saccharin (an average of 7%) was merely loosely bound to a normal urinary constituent so that it failed to extract in the normal manner. SAMB was not detected as such in the urine samples of any of the subjects.

INTRODUCTION

The current widespread concern regarding the safety of many environmental chemicals and the 1969 ban on the unregulated use of cyclamate in foods and beverages have called into question the safety of saccharin, the principal alternative artificial sweetening agent available. This agent has enjoyed extensive use for about 80 years, with a remarkably low incidence of reported adverse effects (Kennedy & Fancher, 1970). Nevertheless, there remains a question whether saccharin gives rise in man to any metabolic products and, if so, whether these products could contribute significantly to its toxicology. The most prominent degradation product expected to be formed in significant amount is that derived by hydrolysis, *o*-sulphamoylbenzoic acid (*o*-SAMB). This compound has been reported to be present in the urine of monkeys receiving saccharin, as has another hydrolytic product, *o*-carboxybenzenesulphonic acid (Pitkin, Andersen, Reynolds & Filer, 1971)‡. However, there is reason to think (Byard, 1972; Byard & Golberg, 1973) that the degradation products

*These studies were supported by Research Grant 2P-01-ES-00226-06 from the National Institute of Environmental Health Sciences, NIH, by the National Institutes of Health Training Grant 2T-01-ES-000103-06, and by FDA Contract 69-7.

†Presented at the 12th Annual Meeting of the Society of Toxicology, New York, March 1973.

‡Although Pitkin *et al.* identified "Compound II" as ammonium *o*-sulphamoylbenzoic acid, they presumably meant ammonium *o*-carboxybenzenesulphonate. That the reference compound as supplied is an ammonium salt is irrelevant, since in urine the acid would not be associated with any specific cation.

reported in such species as the monkey (Pitkin *et al.* 1971) and the rat (Kennedy, Fancher, Calandra & Keller, 1972) could in fact have been derived from a trace of impurity in the radioactive saccharin administered, and not from saccharin itself. The latter authors, however, felt that they had demonstrated satisfactorily the absence of such impurities.

Saccharin is evidently eliminated rapidly from the body (Food Protection Committee, 1955), as would be expected from its highly polar character. Since the experimental studies so far published have used [^{14}C]saccharin, the problem of determining saccharin as such has not arisen. However, for large-scale excretion studies in man it would be patently undesirable to administer the labelled compound except in a very limited way, as might be necessary to prove definitively that all the excretory products were being detected and accounted for. The results of an investigation along these lines will be reported in the next paper of this series.

Two analytical methods have been published recently for the measurement of saccharin. Conacher & O'Brien (1970) described a procedure for the determination of saccharin in soft drinks, and this has provided a basis for the method described below. The Conacher–O'Brien technique depends on the extraction from acidified urine of organic acids present therein, conversion of the acids to their methyl esters, and gas-chromatographic (GLC) analysis of the esters. While the present studies were in progress, the method of Daun (1971) appeared in print. This procedure was applied to the determination of saccharin in a variety of biological materials, but dealt specifically with urine only at the unrealistic levels of 3300–5500 mg/litre. For the present studies, it was necessary to have a precise method not only for the estimation of saccharin at both high (*c.* 1 g/litre) and low (5–10 mg/litre) levels, but also for the estimation of SAMB at the very low concentrations (5–10 mg/litre) projected as possibly being present in the urine samples to be collected in the experiments described below.

EXPERIMENTAL

Experimental design and conduct

Experiment 1. In a preliminary experiment, three male laboratory volunteers took 1·17 g doses of commercial soluble saccharin, estimated to contain the equivalent of 970 mg saccharin. This dose was ingested in a glass of iced tea, and urine samples were collected over chloroform for the periods 0–24 and 24–48 hr thereafter. As a control, these subjects had collected a 24-hr sample for the period just prior to the saccharin administration.

Experiment 2. The procedure for the second experiment, involving six volunteers, differed from the first only in that the dose consisted of 0·5–1 g saccharin dissolved in water at pH 6–7 and made up to about 150 ml as iced tea, and the urine-collection periods were generally 0–8, 8–24, 24–48 and 48–72 hr. Two subjects participated in both experiments, and one of these was tested twice in the second experiment with different doses.

Materials

The solvents used at various stages of the analytical procedures were generally of standard laboratory grade and required no special purification. 1-Naphthyl acetate (m.p. 44–45°C) was a product of the British Drug Houses Ltd., London, saccharin was obtained from Eastman Kodak Co., Rochester, N.Y. (Catalogue no. 38) and *o*-SAMB (m.p. 156–158°C) was obtained from Monsanto Chemical Co., St. Louis, Mo. GLC analysis showed the

sample of *o*-SAMB to contain about 2·0% saccharin. Diazomethane was prepared from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) by the method of McKay (1948), yielding an ethereal solution of which 6 ml was sufficient to methylate completely 1 m-equiv. organic acid.

Analytical methods

Large-scale extraction and methylation of saccharin. To 10 ml urine measured into a 150 or 250 ml screw-capped centrifuge tube were added 0·3 ml 6N-HCl and 90·0 ml ethyl acetate–chloroform (4:1, v/v) mixture. The tube was capped securely, shaken mechanically for 3–4 min and then centrifuged for about 5 min at 1500 rev/min. The aqueous phase was then aspirated as completely as possible, 2–3 g anhydrous Na_2SO_4 was added and the mixture was shaken briefly by hand, after which 80·0 ml of the clear solvent phase was transferred to a 200 ml standard-taper round-bottom flask and 3 ml ethereal diazomethane was added. The flask was stoppered and set aside at room temperature for 3–4 min, and then the solvents were evaporated *in vacuo* in a rotary evaporator. Finally, the residue was taken up in 4·45 ml internal standard solution (160 mg 1-naphthyl acetate in 1000 ml methanol). Of the extract, 1 ml was equivalent to 2 ml urine.

Preparation of saccharin standard. To 20 mg saccharin in a 200 ml standard-taper round-bottom flask, 80 ml ethyl acetate–chloroform (4:1, v/v) mixture was added and the mixture was swirled gently until the saccharin was completely dissolved. After addition of 3 ml ethereal diazomethane, the procedure described above was followed, the esterified residue being taken up in 20 ml internal standard solution (1 ml = 1 mg saccharin).

GLC analysis. A Barber–Colman model 5300 gas chromatograph was used in the early experiments, with a single 6 ft coiled glass (5 mm i.d.) column and a single flame ionization detector. The most satisfactory packing for the analysis (as demonstrated by Dr. T. B. Griffin) was 11% OV17 and QF1 (mixed phase) on 80/100 mesh Gaschrom Q. This material was obtained from Applied Science Laboratories, State College, Pa. For an oven temperature of 150°C and a carrier-gas pressure of 6 psi, the observed retention times were 6·8 min for the internal standard and 12·7 min for *N*-methyl saccharin. These conditions gave a sufficient separation of the latter from methyl hippurate (m.p. 81–83°C; retention time 14 min) to permit the accurate estimation of small amounts of saccharin in the presence of large amounts of hippurate.

While these studies were in progress a Perkin–Elmer model 990 gas chromatograph became available. It was also used in conjunction with the OV17–QF1 packing, and could be operated isothermally, at 170°C. However, it was more convenient to programme the oven temperature as follows: (a) 175°C/8 min; (b) +8°C/min; (c) 205°C/6 min. With the carrier-gas flow rate adjusted to give a retention time of 4·5 min for 1-naphthyl acetate, the observed retention times (in min) were: *N*-methyl saccharin, 8·5; methyl hippurate, 9·2; methyl sulphamoylbenzoate (methyl SAMB), 14·4; *O*-methyl saccharin (Ayça, 1957), 16·5. This separation of saccharin from hippurate was again sufficient.

Calculation of results. The usual analytical sequence was to inject the saccharin (SA) standard, followed by two unknown samples and then a repetition of the standard. The peak heights for the internal standard (IS) and for *N*-methyl saccharin (NMSA) were measured in the usual way to the projected base-line, and the ratio SA/IS was calculated for each sample (for 1 mg saccharin/ml this ratio was usually about 2·0). Since the calibration curves for both the internal standard and saccharin were linear over a very wide range

of concentrations, and 1 ml of extract was equal to 2 ml of urine, the final result was given simply by the formula:

$$\frac{\left[\frac{\text{NMSA}}{\text{IS}}\right]_{\text{unknown}} \times 1000}{\left[\frac{\text{NMSA}}{\text{IS}}\right]_{\text{standard}} \times 2} = \text{mg SA/litre in unknown}$$

in which the term $[\text{NMSA/IS}]_{\text{standard}}$ represents the mean ratio obtained for the standards preceding and following the unknown.

During the analysis the attenuator (usually set at 128 for the internal standard) was adjusted so as to give the maximum possible magnification of the saccharin peak without having the curve go off the paper. The unknown amplitudes were then multiplied by the appropriate factors (from $\frac{1}{16}$ to 4) to put them on the same sensitivity basis as the standard.

Recovery of saccharin from urine. For nine samples prepared to contain 934 mg saccharin/litre (analysis in the Barber–Colman) the recovery (means ± SD) was 96·4 ± 3·2%. For eight samples prepared to contain 732 mg saccharin/litre (analysis in the Perkin–Elmer) the recovery was 97·1 ± 3·0%. For a consideration of recoveries at lower levels of saccharin, see below.

Determination of o-*SAMB.* The extraction and methylation procedure was the same as that described above for saccharin.

GLC analysis. In the Barber–Colman instrument, an oven temperature of 160°C and a carrier-gas pressure of 10 psi gave retention times of 2·4 min for the internal standard and of 12·3 min for methyl SAMB. However, the analysis was more conveniently performed in the Perkin–Elmer, programming the oven temperature as follows: (a) 170°C/12 min; (b) +24°C/min; (c) 200°C/6 min. With this combination both saccharin and *o*-SAMB could be determined on a single chromatogram, since the observed retention times (in min) were: internal standard (adjusted to, by regulating the carrier-gas flow), 6·3; *N*-methyl saccharin, 11·9; methyl hippurate, 12·8; methyl SAMB, 19·1. (For a typical chromatogram see Fig. 1. It may be noted that the methylation of saccharin with diazomethane produced two extraneous peaks clearly related to saccharin. These were peaks D and F. One of these (F) was evidently the *O*-methyl pseudosaccharin observed by Conacher & O'Brien (1970); the nature of the material represented by peak D was not clear.) The combined analysis for saccharin and *o*-SAMB was performed by comparing the responses of the unknown extracts with those of a combined standard solution (for example, per ml: 1-naphthyl acetate, 0·16 mg; saccharin 0·9 mg; *o*-SAMB, 0·1 mg). The peak heights for saccharin and *o*-SAMB were compared to that of the internal standard, and were then evaluated as described on the preceding page. However, the calibration curve for *o*-SAMB was not linear, so the ratio of peak heights (SAMB/IS) decreased from about 2·2 at 1 mg/ml to 0·12 at 0·1 mg/ml. This property of methyl SAMB necessitated calibration of the instrument over the approximate range 0–0·3 mg/ml.

Preparation of o-*SAMB standard.* The procedure used was the same as that described for the preparation of the saccharin standard, the ester residue being taken up in 1 ml of internal standard solution for each mg of *o*-SAMB methylated. Quantities of 100 mg or more of *o*-SAMB could be methylated by refluxing in methanol with a BF_3 catalyst.

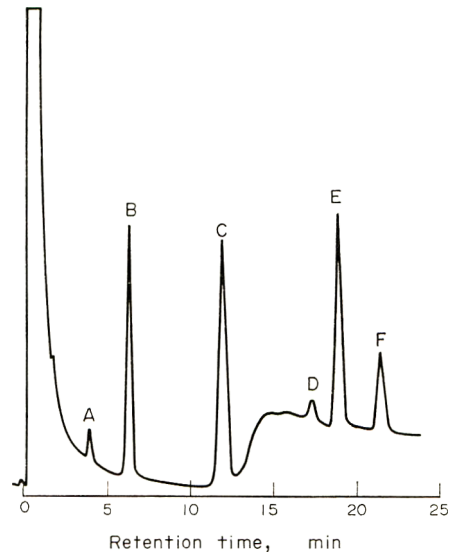


FIG. 1. Gas–liquid chromatogram of a solution prepared by the methylation of equal amounts of saccharin and *o*-SAMB, but estimated to contain the following, as mg/litre: 1-naphthyl acetate, 160 (peak B); *N*-methyl saccharin, 426 (peak C); methyl SAMB, 523 (peak E); *O*-methyl saccharin, 121 (peak F). Peak A represents a reagent artefact, but the nature of peak D is unknown. Temperature programme: (a) 170°C/12 min; (b) +24°C/min; (c) 200°C/12 min.

Recrystallization from 30% methanol gave a product of m.p. 121–122°C (Loev & Kormendy, 1962).

Calculation of results. The procedure was that already described, except that, as noted, the comparison for *o*-SAMB had to be made against standards containing amounts of the same order as were present in the unknowns. Below 0·1 mg/ml, the calibration curve for *o*-SAMB could be assumed to be linear.

Recovery of o-*SAMB from urine.* For concentrations ranging from 10 to 100 mg/litre, the recovery varied irregularly from 83 to 114% (mean $\pm$ SD = 99 $\pm$ 11). At 5 mg/litre the recovery was only 56%, but the response was clearly distinguishable from that of the urine blank. The overall recovery, therefore, was satisfactory enough, but the precision of the method was less than that generally considered desirable. However, for the determination of a compound that has not been detected with certainty in any human urine sample so far tested (following the administration of saccharin) greater precision would have no particular advantage. Since the urine sample used in the recovery experiment contained 732 mg saccharin/litre, it followed that the presence of saccharin did not interfere with the determination of *o*-SAMB (urine blank = zero).

Determination of saccharin at low concentrations. For this analysis slight technical modifications were introduced into the method.

Procedure. To a 5 ml sample of urine in a 40 ml glass-stoppered centrifuge tube 0·5 ml 6 N-HCl and 30·0 ml ethyl acetate–chloroform (1:1, v/v) mixture were added. The mixture was shaken mechanically for 3–4 min, and then centrifuged at 1500 rev/min for about 5 min. After aspiration of the aqueous phase, the solvent phase was dried by shaking with 1–2 g anhydrous Na_2SO_4, and then 25·0 ml of the clear solvent

phase was transferred to a 100 ml standard-taper round-bottom flask. After addition of 1 ml ethereal diazomethane, the procedure used for the larger scale extraction and methylation was followed, the final esterified residue being taken up in 1 ml internal standard solution. For GLC analysis of these extracts, the saccharin standard containing 1 mg/ml was diluted with 11 vols internal standard. For the Perkin–Elmer, the best temperature programme appeared to be: (a) 165°C/16 min; (b) +24°C/min; (c) 215°C/4 min. With the carrier-gas flow rate set to give a retention time of 7 min for the internal standard, the retention time of *N*-methyl saccharin was 13·5 min, and that of methyl hippurate was 15 min, an entirely adequate separation.

Calculation of results. Since 1 ml extract was equal to 4·16 ml urine, and the saccharin standard was equivalent to 83·3 mg/litre, the result was given by the formula:

$$\frac{\left[\frac{\text{NMSA}}{\text{IS}}\right]_{\text{unknown}} \times 20}{\left[\frac{\text{NMSA}}{\text{IS}}\right]_{\text{standard}}} = \text{mg SA/litre in sample}$$

Recovery from urine. In the 3–15 mg/litre range, the recovery (mean ± SD) was 98·6 ± 4·1%.

Determination of saccharin in blood plasma. Although not used in the studies reported in this paper, a method has also been developed for determining saccharin in plasma. This is the same as that used for determining low concentrations of saccharin in urine, except that, since the acidified plasma was difficult to aspirate completely following the extraction, the drying operation with Na_2SO_4 was omitted. Also, the principal interfering substance (of unknown identity) in plasma had a retention time about 0·85 of that of *N*-methyl saccharin, and in order to get a satisfactory separation of these two compounds the temperature programme used was: (a) 190°C/16 min; (b) +24°C/min; (c) 215°C/4 min. The flow rate in this case was adjusted to give a retention time of 8 min for the internal standard, and this in turn gave a retention time of 12·2 min for the interfering compound and of 14·5 min for *N*-methyl saccharin. The temperature programming served only to clear the column completely and rapidly before the next injection was made.

The range of saccharin concentrations studied by this method was 0·5–35 mg/litre. At 0·5 mg/litre, the response was clearly distinguishable from the plasma blank (which was zero) and the percentage recovery was essentially the same over the entire range (mean ± SD = 94 ± 7; n = 13).

Hydrolysis of urine prior to analysis. It is known (DeGarmo, Ashworth, Eaker & Munch, 1952) that alkaline hydrolysis converts saccharin to *o*-SAMB, which may then be determined essentially as described above.

Procedure. Urine (10 ml) and 19 N-NaOH (1 ml) were mixed well in a 15 ml glass tube, which was then put in a boiling water-bath for 2 hr. After cooling to room temperature, the contents of the tube were transferred to a 150 ml separating funnel with the aid of 2–3 ml water, and a drop of phenolphthalein indicator was added. The solution was neutralized with 6 N-HCl, 2 ml phosphate buffer (0·2 M, pH 6·2) was added and the solution was extracted with 50 ml ethyl acetate–chloroform (1:1 v/v), which was discarded. A further 0·5 ml 6 N-HCl was added to the solution, which was extracted thoroughly with 80 ml ethyl acetate, the extract being transferred quantitatively to a 200 ml standard-taper round-bottom flask for evaporation to dryness in

the rotary evaporator. The residue was taken up in 65 ml ethyl acetate, followed shortly by 15 ml chloroform. Ethereal diazomethane (3–4 ml), was added and the flask was stoppered and set aside for 5–10 min, after which the solution was evaporated to dryness in the rotary evaporator. Finally the residue was taken up in 5 ml internal standard solution, for analysis for *o*-SAMB as described earlier (p. 406). It was necessary in this case to run *o*-SAMB standards of the order of 1 mg/ml and on occasion to dilute the unknowns so that they were within the range of the standards.

Calculation of results. This was performed as already described (p. 408). The results were expressed in terms of saccharin by multiplying by 0·905.

Recovery. Four samples of urine known to contain 712 mg saccharin/litre were analysed in this way, giving a recovery of 96·6 $\pm$ 2·3% of the calculated amount of *o*-SAMB.

RESULTS

The data obtained in experiments 1 and 2 are presented in Tables 1 and 2, respectively. In the former experiment, two of the subjects appeared to excrete the dose quantitatively

Table 1. *Excretion of saccharin by three subjects following the ingestion of a 1·17 g dose of the sodium salt*

Subject	Period of collection (hr)	Saccharin excreted		
		Urinary level (mg/litre)	Total in sample* (mg)	Proportion of dose (%)
M	0–24	1170	820	
	24–48	185	148	
	0–48	—	968	99·8
B	0–24	896	922	
	24–48	67	83	
	0–48	—	1005	102·5
A	0–24	785	733	
	24–48	12	9	
	0–48	—	742	76·1
Mean	0–48	—	906	92·8

*Analysis of control samples collected by subjects M, B and A yielded values of 0·5 and 2 mg/24 hr respectively.

as saccharin within 48 hr, but the other subject fell considerably short of this mark. However, it became evident in experiment 2 that none of the subjects (who included B and M from experiment 1) excreted the dose quantitatively as saccharin, even within 72 hr. The maximum saccharin recovered from any subject in the second experiment was 93% of the dose and the minimum was 79%. The bulk of the excretion occurred in the first 24 hr (accounting on average for 81% of the dose, indicating a body half-life of about 10 hr), with all but two of the subjects excreting less than 2% of the dose as saccharin in the 48–72-hr period. Alkaline hydrolysis of the urine samples gave a net yield 7% higher on average

Table 2. *Excretion of saccharin by human volunteers given a single dose ranging from 500 to 1000 mg*

Subject	Dose (mg)	Period of collection (hr)	Saccharin excreted (% of dose)		
			Saccharin	"Combined saccharin"*	Total
B	700	0–8	70·2	4·8	75·0
		8–24	13·7	0	13·7
		24–48	5·6	0	5·6
		48–72	1·9	0·4	2·3
		0–72	90·4	5·2	96·6
G	1000	0–8	49·8	8·7	58·5
		8–24	22·5	1·8	24·3
		24–48	6·7	6·0	12·7
		48–72	0·4	ND	0·4
		0–72	79·4	15·5	95·9
K	700	0–8	71·6	3·8	75·4
		8–24	15·3	0·4	15·7
		24–48	3·2	1·9	5·1
		48–72	0·3	0	0·3
		0–72	90·4	6·1	96·5
L	1000	0–8	68·0	−0·1	67·9
		8–24	21·8	−0·8	21·0
		24–48	0·8	0·5	1·3
		48–72	0·5	ND	0·5
		0–72	91·1	−0·4	90·7
M	500	0–8	62·0	−1·2	60·8
		8–24	20·2	0	20·2
		24–48	11·0	2·8	13·8
		0–48	93·2	1·6	94·8
M	1000	0–8	51·8	5·7	57·5
		8–24	23·2	4·1	27·3
		24–48	12·3	−0·2	12·1
		48–72	2·0	ND	2·0
		0–72	89·3	9·6	98·9
R	950	0–8	52·2	1·5	53·7
		8–24	24·3	4·8	29·1
		24–48	7·7	3·9	11·6
		48–72	0·2	ND	0·2
		0–72	84·4	10·2	94·6
Mean ± SEM		0–8	60·8 ± 3·5	3·3 ± 1·3	64·1 ± 3·3
		8–24	20·1 ± 1·7	1·5 ± 0·8	21·6 ± 2·1
		0–24	80·9 ± 2·6	4·8 ± 1·8	85·7 ± 1·5
		24–48	6·8 ± 1·6	2·1 ± 0·7	8·9 ± 1·8
		0–48	87·6 ± 1·8	6·9 ± 2·2	94·6 ± 0·8
		0–72†	87·5 ± 1·9	7·8 ± 2·3	95·5 ± 1·1

ND = Not determined

*Increased amount found on alkaline hydrolysis.
†Omitting subject M, 500 mg dose.

than that calculated from the saccharin output. This brought the mean total 72-hr recovery up to 95·5 ± 1·1% of the dose.

In no subject was even a trace of free *o*-SAMB found in the urine, except perhaps in B. In experiment 1, his 0–24-hr sample appeared to contain *o*-SAMB in an amount not exceeding 0·2% of the dose, but the chromatogram in question was not typical and the identification was very doubtful. In experiment 2, samples from subject B all appeared to contain some *o*-SAMB, the 0–72 hr total being approximately 20 mg. However, his control sample, collected prior to the administration of saccharin, contained a very large amount of a substance having the same retention time as methyl SAMB. Hence the possibility that subject B converted saccharin to *o*-SAMB could virtually be discounted.

DISCUSSION

Esterification methods

A considerable number of methylating agents has been studied by Conacher & O'Brien (1970), by Daun (1971) and by the present authors. Both Conacher & O'Brien (1970) and the present authors eventually concluded that, considering all factors, diazomethane was the best available agent for the intended application. Daun (1971) also examined the possibility of using diazomethane, but abandoned it on the ground that a mixture containing both the *N*-methyl and *O*-methyl esters of saccharin was produced. This appeared to require the evaluation of two separate chromatographic peaks. Consequently, Daun adopted the method of refluxing saccharin in dimethylsulphoxide with methyl iodide. Thereafter, dilution with water, extraction with $CHCl_3$ and evaporation of the solvent were required. In the opinion of the present authors, the need to measure a second peak is a minor inconvenience in comparison with the more involved esterification procedure proposed by Daun. Moreover, we concur with Conacher & O'Brien in the finding not only that the *O*-methyl saccharin peak is relatively small but that the ratio of peaks (*N*-methyl/*O*-methyl, based on areas) remains constant regardless of the amount of saccharin being methylated. We have found that this ratio is not greatly altered by the volume of ethereal diazomethane added over the range 1–4 ml (all of these volumes providing an excess of the reagent). However, less of the extraneous peak D (see Fig. 1) is formed in the 4:1 than in the 1:1 solvent mixture, and less is formed in each of these mixtures as the volume of diazomethane increases. The optimal conversion of saccharin to *N*-methyl saccharin thus far achieved, therefore, has been with the 4:1 solvent mixture, and with the addition of 3–4 ml of ethereal diazomethane. The prominence of peak D also appears related in some way to the conditions of chromatography, even taking into account the area under the curves, since with some temperature programmes it represents as much as 3–4% of the total saccharin being methylated, while in others it represents as little as 1%. Also, its amplitude appears to increase during storage of the samples at −20°C. Peak F, on the other hand, represents such a constant factor that (except for the lower sensitivity) it could be used almost as satisfactorily as peak C as a measure of the total saccharin present.

Analysis of plasma

It is evident, as shown on p. 408, that the procedure for the determination of saccharin in plasma does not need to be as elaborate and involved as that described by Daun (1971). Even concentrations as low as 0·5 mg/litre are detectable by the method described in this

paper, and can be determined with fair accuracy (probably ±25%). However, Daun used a considerably smaller sample (1 ml whole blood), and the 5 ml of plasma required for the method described here might not always be available. Although Daun reported analyses of blood (from rats) containing as little as 0·4 mg saccharin/litre, the contribution represented by his method is difficult to assess, since no data indicating its precision or the ultimate level of detectability were provided.

Experimental data

The results of experiment 1 suggested that the excretory and metabolic pattern of saccharin in man was very similar to that observed in animals (Byard, 1972; Byard & Golberg, 1973; Minegishi, Asahina & Yamaha, 1972), with saccharin being rapidly excreted in unchanged form. Although one of the three subjects had apparently excreted only about 75% of the dose in 48 hr, it was tempting to conclude at the time that a part of his 0–24-hr sample had not been collected.

The results of experiment 2 cast some doubt on this simple concept of the human metabolism of saccharin. Now none of the six subjects was found to excrete the dose quantitatively in 72 hr, in spite of the fact that two of them had appeared to do so within 48 hr in the course of experiment 1. The alkaline hydrolysis method was therefore developed as a check, to determine whether any of the missing saccharin could be accounted for in this way. It was found that the hydrolysis as performed did appear to release a small amount (0–16% of the dose) of either saccharin or *o*-SAMB from some type of combination, and it was clear that the derivative released did not involve the transformation of saccharin beyond the stage of *o*-SAMB.

The principal possibilities for the additional amount found on hydrolysis appeared to be that it could be present as *o*-sulphamoylhippuric acid, as *o*-SAMB ester glucuronide, or simply as saccharin loosely bound to some normal urinary constituent (and therefore showing a modified degree of extractability). Considering these possibilities in order, no specific precedent for the conjugation of *o*-SAMB with either glycine or glucuronic acid exists. Thus, in their study of the metabolism of homosulphanilamide, Hartles & Williams (1947), noted its conversion to *p*-SAMB, but there was no evidence that the latter subsequently conjugated with either glycine or glucuronic acid. It seems reasonable to suppose that *o*-SAMB would be metabolized in the same manner as the *p*-analogue, but, in any case, Minegishi *et al.* (1972) have specifically observed that over 90% of both *o*- and *p*-SAMB are excreted unchanged by rats. Further, it seems unlikely that *o*-sulphamoylhippurate could be formed in the liver and be excreted in the urine by man without the appearance of free *o*-SAMB in the urine.

This problem was studied in further detail in some of the urine samples, which gave particularly large increments of recovery on alkaline hydrolysis. After extended digestion with β-glucuronidase at pH 5 (37°C), the samples were extracted and analysed by the procedure described for *o*-SAMB determinations (p. 406). No increase in free saccharin or *o*-SAMB was detectable. The possibility that either compound was converted to a glucuronide was therefore eliminated, but the chance remained that *o*-SAMB was formed and converted rapidly to *o*-sulphamoyl hippuric acid. Treatment of the 0–8 hr sample (experiment 2) from subject B at pH 3 for 1 hr at 100°C appeared to increase the recovery as saccharin from 70·2% of the dose to 75%, while the same treatment of subject B's 8–24-hr sample yielded no increase. This observation suggested that part of the saccharin in the 0–8-hr sample was present as saccharin in some sort of loose chemical combination. It

could, therefore, represent an artefact of the type observed by Miller, Crawford, Sonders & Cardinal (1966) following the administration of cyclamate to a dog. A further possibly significant observation was the fact that when samples showing a considerable increase in recovery on alkaline hydrolysis were analysed again after storage for 1–8 wk at −20°C, the gap between free and total saccharin almost invariably decreased. A notable exception was the 24–48-hr sample from subject G, which showed no change from the values recorded in Table 2 when analysed again after storage at −20°C for 9 months.

It is of some interest to compare the rate of urinary excretion of total saccharin in man with that of [^{14}C]saccharin in the monkey (Pitkin *et al.* 1971), since the doses were comparable (10 mg/kg). In man the 72-hr excretion for six subjects was 95·5 ± 1·1%, while in three monkeys it was 97·4 ± 0·5% of the dose. Therefore, in effect, it may be stated that these two species eliminate saccharin at substantially the same rate.

REFERENCES

Ayça, E. (1957). Proton-methylation with diazomethane, studied on saccharin. *Istanb. Univ. Fen Fak. Mecm.* (*C*) **22,** 383.

Byard, J. L. (1972). Observations on the metabolism of saccharin. *Toxic. appl. Pharmac.* **22,** 291.

Byard, J. L. & Golberg, L. (1973). The metabolism of saccharin in laboratory animals. *Fd Cosmet. Toxicol.* **11,** 391.

Conacher, H. B. S. & O'Brien, R. C. (1970). Gas–liquid chromatographic determination of saccharin in soft drinks. *J. Ass. off. analyt. Chem.* **53,** 1117.

Daun, R. J. (1971). Determination of saccharin in biological materials by gas–liquid chromatography. *J. Ass. off. analyt. Chem.* **54,** 1140.

DeGarmo, O., Ashworth, G. W., Eaker, C. M. & Munch, R. H. (1952). Hydrolytic stability of saccharin. *J. Am. pharm. Ass.* **41,** 17.

Food Protection Committee (1955). *The Safety of Artificial Sweeteners for Use in Foods.* A Report by the Food Protection Committee of the Food and Nutrition Board. National Academy of Sciences-National Research Council Publ. 386. Washington, D.C.

Hartles, R. L. & Williams, R. T. (1947). The metabolism of sulphonamides. 4. The relation of the metabolic fate of ambamide (Marfanil) and V 335 to their lack of systemic antibacterial activity. *Biochem. J.* **41,** 206.

Kennedy, G. R. & Fancher, O. E. (1970). In *Safety of Saccharin for Use in Foods.* National Academy of Sciences–National Research Council Report to the Food and Drug Administration, p. 51.

Kennedy, G. R., Fancher, O. E., Calandra, J. C. & Keller, R. E. (1972). Metabolic fate of saccharin in the albino rat. *Fd Cosmet. Toxicol.* **10,** 143.

Loev, B. & Kormendy, Minerva (1962). 2-Sulfobenzoic acid esters. I. 2-Sulfamyl derivatives. *J. org. Chem.* **27,** 1703.

McKay, A. F. (1948). A new method of preparation of diazomethane. *J. Am. chem. Soc.* **70,** 1974.

Miller, J. P., Crawford, L. E. M., Sonders, R. C. & Cardinal, E. V. (1966). Distribution and excretion of ^{14}C-cyclamate sodium in animals. *Biochem. biophys. Res. Commun.* **25,** 153.

Minegishi, K.-I., Asahina, M. & Yamaha, T. (1972). The metabolism of saccharin and related compounds in rats and guinea pigs. *Chem. pharm. Bull., Tokyo* **20,** 1351.

Pitkin, R. M., Andersen, D. W., Reynolds, W. Ann & Filer, L. J., Jr. (1971). Saccharin metabolism in *Macaca mulatta. Proc. Soc. exp. Biol. Med.* **137,** 803.

L'excrétion et le métabolisme de la saccharine chez l'homme. I. Méthodes de recherche et résultats préliminaires

Résumé—Des méthodes de détermination de la saccharine et de son métabolite le plus probable (l'acide *o*-sulfamoylbenzoïque; *o*-SAMB) dans l'urine ont été réalisées et appliquées à l'étude du métabolisme de la saccharine chez l'homme. Trois sujets ayant reçu des doses de 1 g, on a constaté que deux d'entre eux l'excrétaient quantitativement par la voie urinaire, dans les 48 h et sous une forme inchangée. Dans une expérience ultérieure, plus détaillée, portant sur six sujets et comportant l'administration d'une dose allant de 0,5–1 g, on a constaté toutefois qu'aucun sujet n'excrétait quantitativement la dose sous forme de saccharine dans les 72 h. L'hydrolyse alcaline des échantillons d'urine, effectuée dans des conditions connues pour convertir quantitativement la saccharine en *o*-SAMB, a fait augmenter jusqu'à 91–99% les récupérations totales de la dose. On n'a pas pu établir clairement quelle forme de saccharine était présente pour rendre compte de la récupération plus marquée après l'hydrolyse alcaline. Il est possible que la saccharine se soit hydrolysée en *o*-SAMB *in vivo*, puis conjuguée avec la glycine, mais on a aussi envisagé la possibilité qu'une partie de la saccharine (en moyenne 7%) n'était fixée que d'une manière fort lâche au composant normal de l'urine, de sorte qu'elle ne se prêtait pas à l'extraction normale. Le SAMB n'a été décelé en tant que tel dans les échantillons d'urine d'aucun des sujets.

Die Ausscheidung und der Stoffwechsel von Saccharin beim Menschen. I. Untersuchungsmethoden und vorläufige Ergebnisse

Zusammenfassung—Methoden für die Bestimmung von Saccharin und seinem wahrscheinlichsten Abbauprodukt (*o*-Sulfamoylbenzoesäure; *o*-SAMB) im Urin wurden entwickelt und bei einer Untersuchung des Stoffwechsels von Saccharin beim Menschen angewendet. Wurde Saccharin in Dosen von 1 g an drei Versuchspersonen verabreicht, dann schien es quantitativ in unveränderter Form im Urin von zwei der Versuchspersonen innerhalb von 48 Stunden ausgeschieden zu werden. Bei einer späteren und eingehenderen Untersuchung an sechs Versuchspersonen mit Verabreichung einer Dosis im Bereich 0,5–1 g wurde jedoch festgestellt, dass keine die Dosis innerhalb von 72 Stunden quantitativ als Saccharin ausschied. Die alkalische Hydrolyse der Urinprobe unter Bedingungen, die bekanntermassen Saccharin quantitativ in *o*-SAMB umwandeln, erhöhte die Gesamtwiedergewinnung auf 91–99% der Dosis. Es wurde nicht definitiv festgestellt, welche Form von Saccharin vorlag, um die vermehrte Wiedergewinnung nach alkalischer Hydrolyse zu erklären. Die Hydrolyse von Saccharin zu *o*-SAMB *in vivo*, gefolgt von Konjugation mit Glycin, war eine Möglichkeit, es wurde aber ebenfalls für möglich gehalten, dass ein Teil des Saccharins (durchschnittlich 7%) nur lose an einen normalen Urinbestandteil gebunden war, so dass er sich nicht auf normale Weise extrahieren liess. SAMB wurde als solche nicht in den Urinproben einer der Versuchspersonen entdeckt.

Fd Cosmet. Toxicol. Vol. 11, pp. 415–432. Pergamon Press 1972. Printed in Great Britain.

The Toxicology of Dieldrin (HEOD*). I. Long-term Oral Toxicity Studies in Mice

A. I. T. WALKER, E. THORPE and D. E. STEVENSON

Shell Research Limited, Tunstall Laboratory, Sittingbourne, Kent, England

(*Received 25 November 1971*)

Abstract—Two studies comprising five long-term oral toxicity experiments have been completed, in which dieldrin (purity >99% HEOD), *p,p'*-DDT (purity >99·5%) and a combination of these two compounds have been fed to CF1 mice at various dietary concentrations. The interaction between the method of diet sterilization and the development of liver changes, and the behaviour of the liver lesion after restoration to the basal diet were also studied. In a positive control group fed 600 ppm 4-amino-2,3-dimethylazobenzene (ADAB), 61% of the males developed sarcomas while pathological changes in the liver were the predominant lesions in the females. No signs of ill-health were associated with the feeding of dieldrin at levels of 10 ppm or less, or of DDT at levels at or below 100 ppm.

In mice fed dieldrin, liver enlargement was detectable after 9 months following treatment with 10 ppm, after 19 months with 5 ppm and after 23 months with 2·5 ppm and was associated in all three groups with a decrease in survival rate. At intakes of 1·25 and 1 ppm dieldrin, no enlargement was detected clinically and survival was not affected. With DDT, liver enlargement was detected in the 100-ppm group after 16 months and morbidity was increased. In the 50-ppm group, enlargement occurred after 24 months, but morbidity was not affected. The method of diet sterilization did not influence the development of liver lesions.

Liver lesions observed in all the groups, including controls, were classed as type (a), a simple nodular growth of liver parenchymal cells, and type (b), areas of papilliform and adenoid growth of tumour cells, sometimes accompanied in both control and treated animals by metastases to the lungs. In many animals the two types of lesion were contiguous. An increased incidence of liver lesions was evident in the treated groups. The incidence of other tumours was increased in ADAB-treated mice but not in the dieldrin or DDT groups.

INTRODUCTION

Studies in dogs and rats (Walker, Stevenson, Robinson, Thorpe & Roberts, 1969), monkeys (A. S. Wright, personal communication 1971) and man (Jager, 1970) have failed to provide evidence of any carcinogenic or tumorigenic activity by aldrin and dieldrin. However, Annau (1953) reported that in mice aldrin increased liver parenchyma, and he investigated whether this proliferative process was similar to that elicited by malignancy or gestation. He quoted previous workers who had shown that both RNA and DNA were increased in malignant tumours in mice and also in the liver parenchyma of the tumour-bearing host. During exposure to aldrin an increase in DNA, but not in RNA, was found and Annau (1953) concluded that the increased liver mass induced by exposure to aldrin differed from that associated with malignancy.

Davis & Fitzhugh (1962) reported two experiments in mice. The first was inconclusive because the majority of animals were not available for pathological examination, but in the

*HEOD (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*exo*-1,4-*endo*-5,8-dimethanonaphthalene) is the major constituent of dieldrin.

second the feeding of 10 ppm dieldrin shortened the lifespan of C3HeB/Fe mice by 2 months and increased the incidence of hepatic tumours. Song & Harville (1964) briefly reported experiments in which aldrin and dieldrin were fed to C3HeB/Fe and CBA/J mice. Unfortunately, only ten control animals were used, compared with treatment groups of 55, so the significance of the neoplasia reported in a few mice cannot be assessed. Keplinger, Deichmann & Sala (1968), investigating the effects of dieldrin on reproduction in Swiss mice, did not report any increase in liver-tumour incidence, a result that may indicate a possible strain variation in response.

Hodge, Boyce, Deichmann & Kraybill (1967) summarized data available up to 1964 on the toxicology of dieldrin. They recommended further investigation of the carcinogenicity of dieldrin, emphasizing some important criteria, namely the use of adequate numbers of experimental and control animals, their general good health and longevity, inclusion of full histological studies and the planning of a test of sufficient duration to be consistent with the potential carcinogenicity of the agent.

A series of experiments based on these recommendations was initiated using specific-pathogen-free mice. Two studies comprising five experiments were carried out between May 1965 and January 1970 and are reported in this paper.

In the first study (Study 1) the mice received diets containing 0, 0·1, 1·0 or 10 ppm dieldrin for 132 wk. As a result of the clinical findings obtained during the first 18 months of this study, namely the development of palpable liver enlargements and a reduction in lifespan in the mice receiving 10 ppm dieldrin, an extension of the study appeared desirable. This extension (Study 2) comprised four experiments and investigated the following aspects:

(1) The existence of a dose–response relationship between the intake of dieldrin and the formation of liver tumours (Experiment 2.1).

(2) The interaction between the method of diet sterilization and the development of liver changes in dieldrin-treated mice (Experiment 2.2). All mice used at Tunstall are specific-pathogen-free and receive diet sterilized by ethylene oxide (Charles, Stevenson & Walker, 1965). The possible interaction between residues of 2-chloroethanol found in the diet after sterilization and dieldrin in producing liver changes was studied, using unsterilized diet and diet sterilized by γ-irradiation.

(3) Relationship between DDT, dieldrin and the development of liver changes (Experiment 2.3).

(4) Hepatic changes following exposure to dieldrin for limited periods of time (Experiment 2.4).

EXPERIMENTAL

Materials. Recrystallized dieldrin (purity > 99% HEOD) was supplied by Woodstock Agricultural Research Centre, Sittingbourne, Kent, *p,p'*-DDT (purity >99·5%, twice recrystallized from methanol) was prepared in the Tunstall Laboratory, Sittingbourne, Kent, and 4-amino-2,3-dimethylazobenzene (ADAB) was supplied by Eastman Kodak Inc., New York, USA. To compensate for any reduction in nutritional quality following sterilization, the diet was supplemented by a 3% addition of a protein–vitamin mixture ('Complan', Glaxo Laboratories Ltd.).

Animals. In this study, 3200 mice of Carworth Farm No. 1 strain were used, bred and maintained under specific-pathogen-free conditions (Walker & Poppleton, 1967).

Experimental design and observations

Study 1. Diets containing nominally 0, 0·1, 1·0 and 10 ppm dieldrin and with actual concentrations of 0·010, 0·100, 0·986 and 9·830 ppm were fed to groups of 600, 250, 250 and 400 mice, respectively, divided equally according to sex. Other organochlorine compounds found in the diet were *p,p'*-DDT (0·018–0·026 ppm), *p,p'*-DDE (0·004–0·009 ppm) and γ-BHC (0·03–0·05 ppm). ADAB in an actual concentration of 600 ppm was given as a positive control to a group of 25 males and 25 females.

Because of the large numbers of animals involved, the mice were placed on the trial in weekly batches over a period of 10 wk.

Weanling mice aged 3 wk were caged together by litters. Each mouse in a litter was earmarked and then the numbers, sex and earmarks of the mice in each litter were recorded so that the distribution of litters within the trial was known. The mice were then allocated at random to a polypropylene mouse box, so that each box eventually contained five mice of one sex. Boxes were randomly allocated to treatments, approximately in proportion to the size of the final treatment groups. The mice were housed together to accustom them to the change from the breeding to the experimental unit. The following week, the pretreatment body weights were measured and the mice were fed the appropriate treated diet (Diet 86 powder, Scientific Products Farm, Ash, Kent). After 6 wk of exposure, the mice were separated into individual boxes and randomly distributed throughout the experimental room.

The males and females receiving dieldrin were kept in separate experimental rooms while those fed ADAB were in a further room to avoid any cross-contamination. Feeding of ADAB was discontinued when the first hepatoma was found in the exposed animals.

The health and behaviour of all the animals were observed daily. Weekly abdominal palpation of the control, 10 ppm and ADAB groups was started at wk 16 to detect the presence of intra-abdominal masses. When such a mass was detected the animal was palpated twice weekly and it was killed when the enlargement was considered to be detrimental to health. This procedure and individual caging were adopted to ensure that as many mice as possible were available for autopsy, at which a gross dissection of each mouse was made and tissue sections of brain, heart, lungs, liver, kidney and testes or ovary and of any macroscopic lesion were prepared and stained with haematoxylin and eosin.

The cumulative percentage morbidity of the various groups was calculated on the basis of the numbers of mice found dead, showing signs of ill-health and sent for autopsy, or sent for autopsy because of the size of the intra-abdominal mass, as discussed above.

Study 2. The same procedures and observations as described for Study 1 were used for the following four experiments, all carried out on groups with equal numbers of males and females:

Experiment 2.1. Dose–response relationship. Groups of 60 mice received ethylene oxide-sterilized diets containing 1·25, 2·5, 5, 10 or 20 ppm dieldrin for 128 wk. The control group comprised 156 animals.

Experiment 2.2. Diet sterilization. The diets fed contained 0 or 10 ppm dieldrin. Groups of 60 mice received γ-irradiated diets for 128 wk. Groups of 48 mice were given γ-irradiated diet and litter for 110 wk or unsterilized diet and litter for 104 wk.

Experiment 2.3. DDT and dieldrin. Ethylene oxide-sterilized diets containing either a 5/50 ppm mixture of dieldrin and DDT or 50 or 100 ppm DDT were given to groups of 64 mice for 112 wk. The control group consisted of 96 mice. A smaller group of 20 animals received 200 ppm DDT for 128 wk.

Experiment 2.4. Limited exposure. Diets containing 10 ppm dieldrin were given to groups of 58 mice for varying periods of time up to 64 wk. The control group comprised 156 animals. After a prescribed period, the treated diet was withdrawn, five treated and five control animals of each sex were killed and autopsied and the remaining animals were fed control diet for the rest of the 104-wk experiment.

RESULTS

Clinical observations

Study 1. During the first 9 months of the experiment, the health and behaviour of the mice were not affected by the dieldrin treatment. The commonest finding in both treated and control animals during this period was lymphosarcoma, a condition often present in mice to an extent varying with the strain (Salaman, 1967). Affected animals showed signs of ill-health, such as loss of weight, roughened coat and ascites in males.

After 9 months, intra-abdominal masses were palpated in both males and females receiving 10 ppm dieldrin, without any evidence of ill-health. Half the males and females receiving 10 ppm dieldrin had died or been killed by month 15 but this stage was reached in the control group only at months 20–24 (Fig. 1). Palpable masses were never detected in the mice given 0·1 or 1 ppm and the lifespan of these animals was similar to that of the controls (Fig. 1).

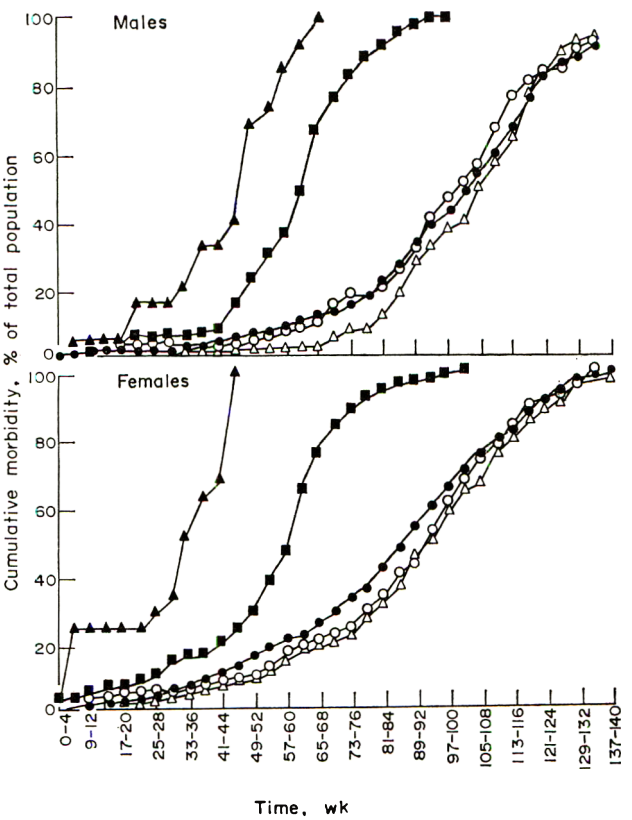


FIG. 1. Cumulative morbidity of mice fed dieldrin at levels of 0 (●), 0·1 (○), 1·0 (△) or 10 (■) ppm or ADAB (▲) at 600 ppm (Study 1).

All mice receiving ADAB were dead by month 15 of the experiment, although the feeding of ADAB had ceased at month 6 when the first intra-abdominal mass was detected.

Experiment 2.1. Dose–response relationship. In the group given 20 ppm dieldrin, about 25% of the males and nearly 50% of the females died during the first 3 months of the experiment (Fig. 2). Most of the affected animals showed signs of dieldrin intoxication, such as body tremors and convulsions. Liver enlargements were detected after wk 36 and all the mice were sent for autopsy within 12 months.

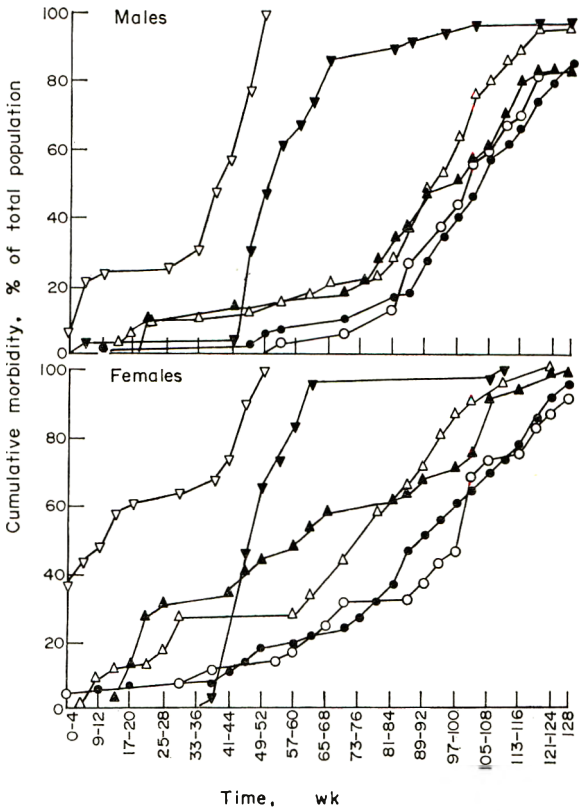


FIG. 2. Cumulative morbidity of mice fed dieldrin at levels of 0 (●), 1·25 (○), 2·5 (▲), 5 (△), 10 (▼) or 20 (▽) ppm (Experiment 2.1: Dose–response relationship).

In the 10-ppm group, intra-abdominal masses were detected after wk 40 and most of the mice were sent for autopsy by 16–17 months, this finding being similar to that of Study 1. Palpable masses were detected in the 5-ppm group after wk 75 and in the 2·5-ppm animals after wk 100. The morbidity of these groups was slightly increased (Fig. 2).

In the 1·25-ppm group, liver enlargements were not palpated, their morbidity being similar to that of the controls. This result was similar to that found in Study 1 in the 1-ppm animals. No signs of dieldrin intoxication were seen at intakes of 10 ppm or below.

Experiment 2.2. Diet sterilization. Liver enlargements were detected between wk 40 and 50 in all the groups given 10 ppm dieldrin. Their development was not affected by the method of diet sterilization, the overall pattern (Fig. 3) being similar to that found in Experiment 2.1 and Study 1.

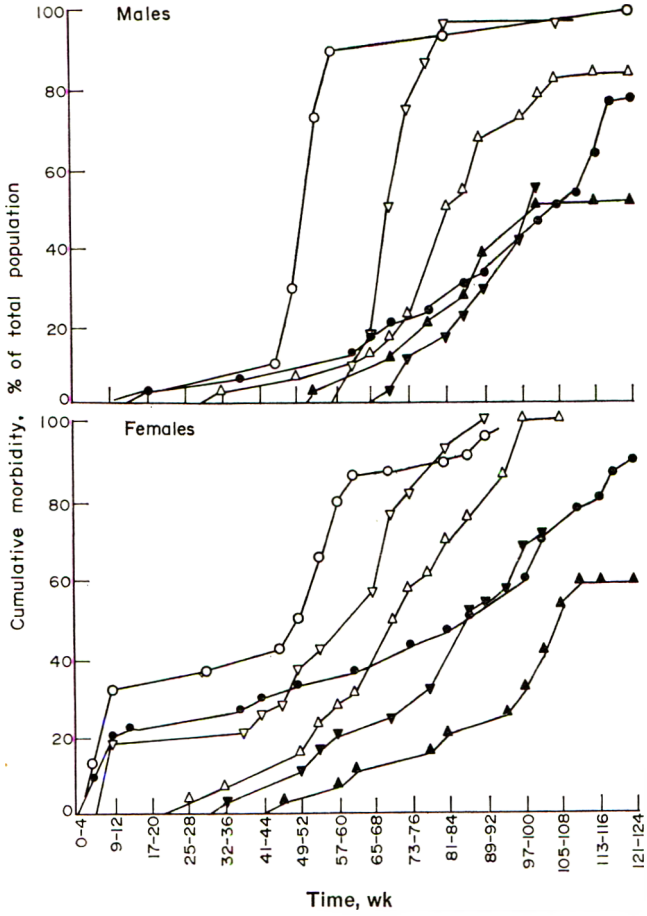


FIG. 3. Cumulative morbidity of mice fed dieldrin at levels of 0 (●,▲,▼) or 10 (○,△,▽) ppm together with γ-irradiated diet (●,○), γ-irradiated diet and litter (▲,△) or unsterilized diet and litter (▼,▽) (Experiment 2.2: Diet sterilization).

Experiment 2.3. DDT and dieldrin. The small number of mice receiving 200 ppm DDT were the survivors of a larger group. Originally, diets of 10/200 ppm dieldrin/DDT and 200 ppm DDT were fed, but after 4 wk 30% of the 10/200-ppm males and females and 25% of the 200-ppm females died. The study was ended and the diets containing 5/50 ppm dieldrin/DDT and 50 and 100 ppm DDT were introduced. In the 200-ppm group, intra-abdominal masses were detected after wk 65. One male survived the 128-wk experimental period, but all females had been sent for autopsy by wk 68.

In animals given 5/50 ppm dieldrin/DDT or 100 ppm DDT, liver enlargements were detected after wk 65. Morbidity was increased in the males of both these groups (Fig. 4) but in the females of the 5/50-ppm group only. At an intake of 50 ppm DDT, intra-abdominal masses were first palpated after wk 96. The morbidity of this group was similar to that of the controls.

Experiment 2.4. Limited exposure. Liver enlargements were detected after wk 60 in only six male and two female mice of the group receiving 10 ppm dieldrin for 64 wk. The enlargements did not appear to increase in size after withdrawal of this dieldrin diet. No en-

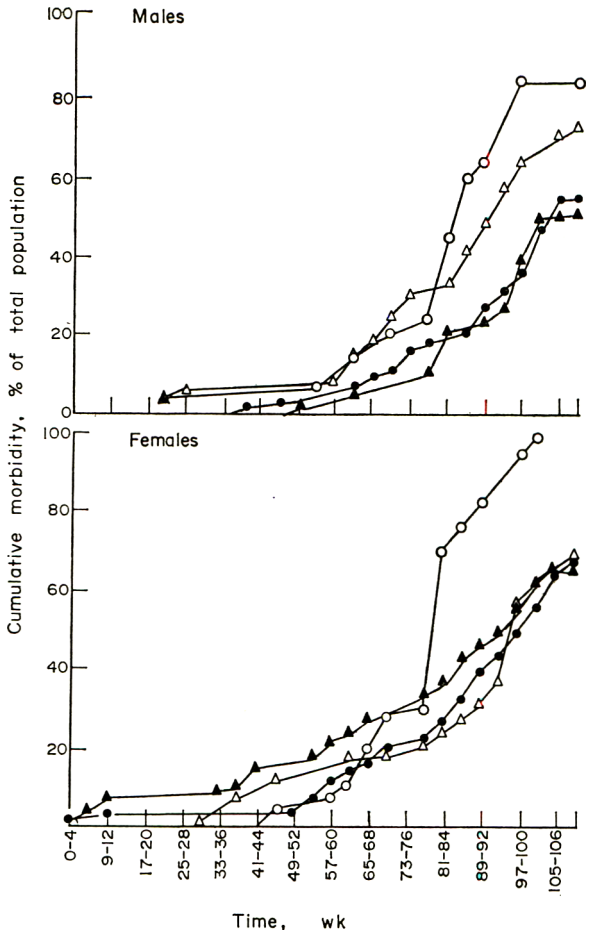


FIG. 4. Cumulative morbidity of mice fed 0 (●), 50 (▲) or 100 (△) ppm DDT or 5/50 ppm dieldrin/DDT (○) (Experiment 2.3: DDT and dieldrin).

largements were palpated in any of the other groups and the morbidity of the various groups was similar in this experiment (Fig. 5). No explanation can be offered for the delay in development of palpable liver enlargements in this experiment, in which enlargements were first detected after wk 60 instead of in the 40–50 wk period common to the other experiments.

Liver pathology

Types of tumour. The main lesions discussed in this paper were associated with the liver. Descriptions of the histology of the two types of liver parenchymal-cell tumour seen in these CF1 mice are relevant to this study of the hepatotoxicity of dieldrin.

The first type of growth, type (a), was composed of solid cords of closely packed parenchymal cells with a morphology and staining affinity little different from the rest of the parenchyma (Fig. 6). The mitotic activity of the cells in the tumour was only occasionally increased and the growth of the lesion appeared to be by expansion, compressing the liver

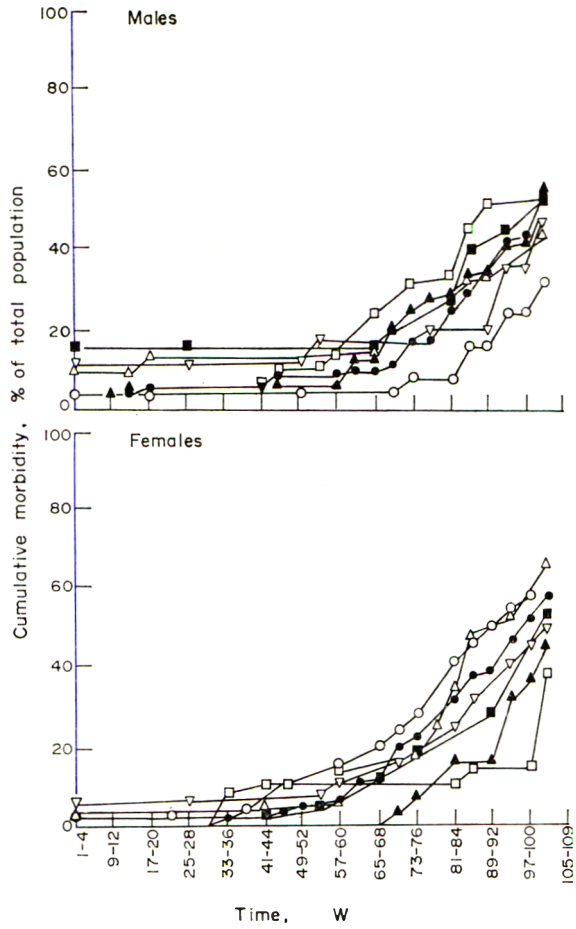


FIG. 5. Cumulative morbidity of mice fed 10 ppm dieldrin for 0 (●), 2 (○), 4 (▲), 8 (▽), 16 (■), 32 (□) or 64 (△) wk (Experiment 2.4: Limited exposure).

cells at the periphery. No fibrous capsule was seen. These growths were single or multiple, ranging in size from a lobule or less to massive structures involving an entire liver lobe. Livers containing one or more of such lesions did not show sufficient fibrosis to support a diagnosis that these growths were hyperplastic or regenerative nodules. Since a clear-cut distinction was not possible between a hyperplastic nodule and a benign hepatoma on cell morphology or size of the growth only, these growths were classified as benign tumours, and are referred to as type (a) tumours in subsequent tables.

The second type of lesion derived from liver parenchymal cells, type (b), was uncommon in control mice. A much more abnormal structure was present (Fig. 7) with areas of cells proliferating in confluent sheets and often with foci of necrosis. These lesions were distinguished from the previous type of growth by the presence of areas of papilliform and adenoid formations of liver cells with wide and irregular vascular channels within the growth. Nuclear abnormalities found in the lesion included a variable increase in size with hyperchromasia, enlarged nucleoli and eosinophilic inclusions. The mitotic activity of the tumour cells was frequently increased and multinucleate forms were seen. Cytoplasmic changes

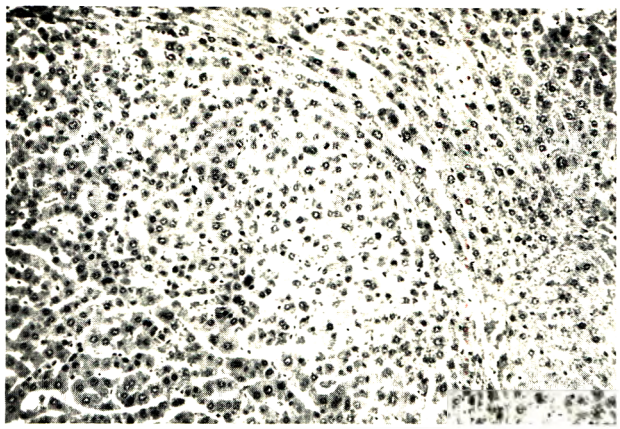


FIG. 6. Type (a) hepatoma in CF1 mouse. Haematoxylin and eosin ×530.

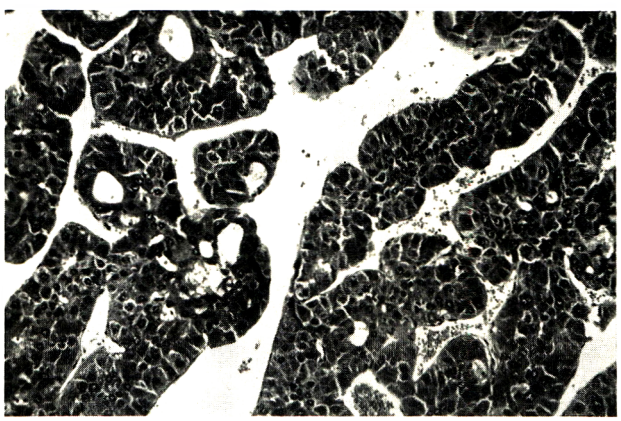


FIG. 7. An area of papilliform and adenoid growth pattern in a type (b) hepatoma in a CF1 mouse. Haematoxylin and eosin ×530.

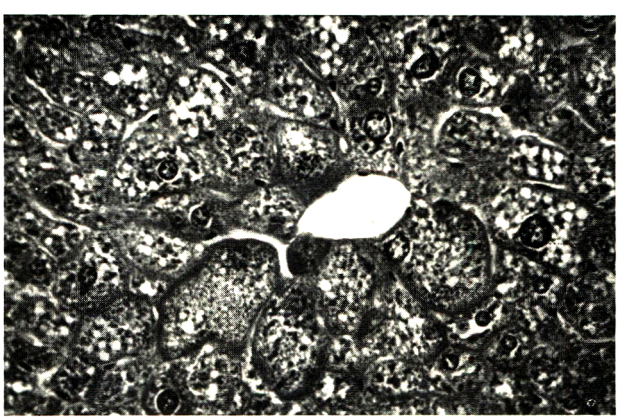


FIG. 8. Nuclear and cytoplasmic enlargement in centrilobular cells from the liver of a mouse fed 10 ppm dieldrin for 30 days. Haematoxylin and eosin ×2100.

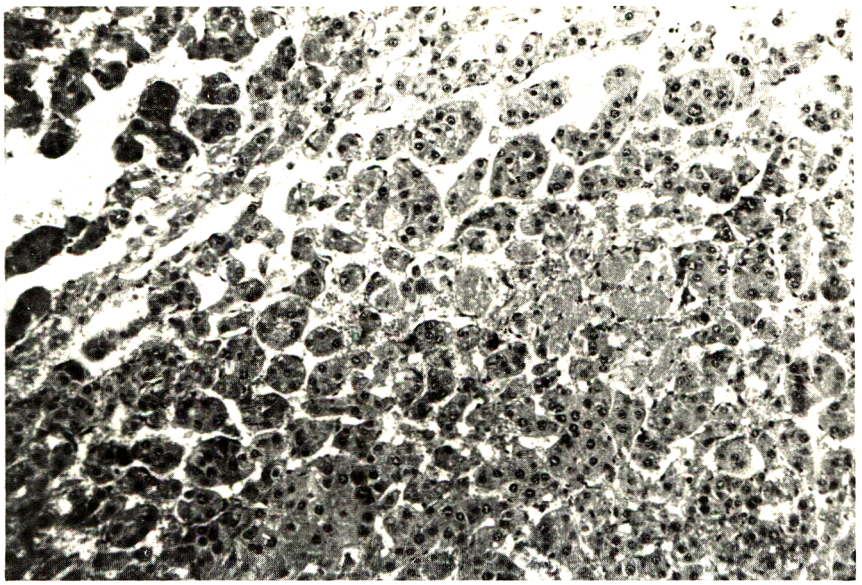


FIG. 9. Type (b) tumour from an untreated male mouse. Haematoxylin and eosin × 530.

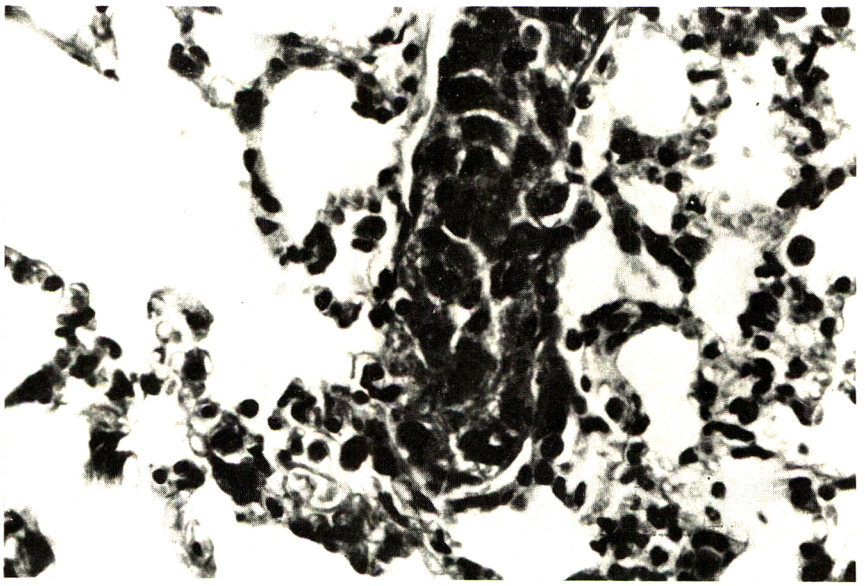


FIG. 10. Embolus of liver tumour cells in a pulmonary vessel from same mouse as Fig. 9. Haematoxylin and eosin × 2100.

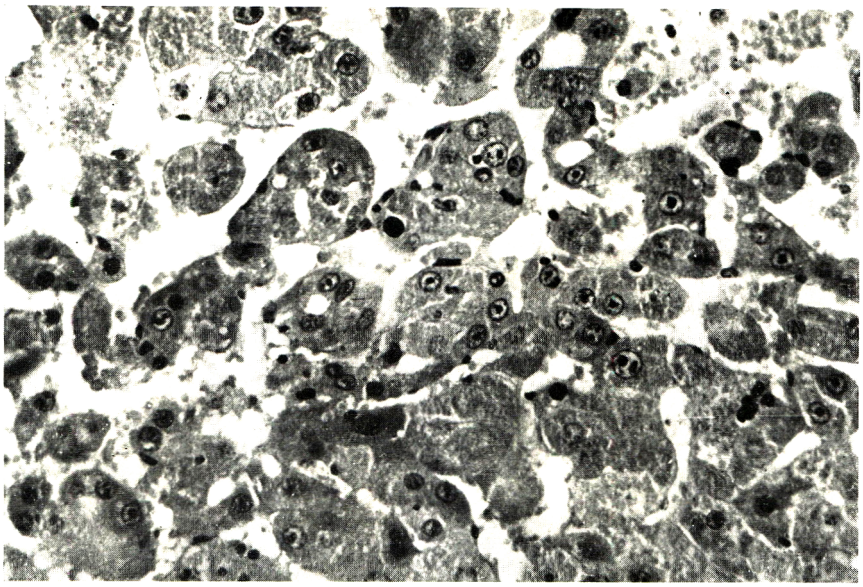


FIG. 11. Type (b) tumour from mouse fed 2·5 ppm dieldrin. Haematoxylin and eosin ×1325.

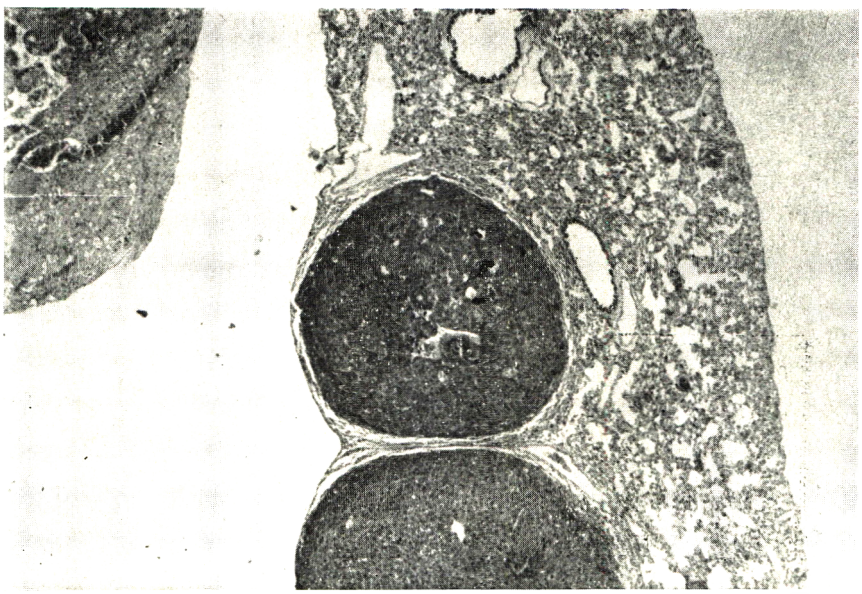


FIG. 12. Pulmonary metastases of the hepatoma shown in Fig. 11. Haematoxylin and eosin ×132.

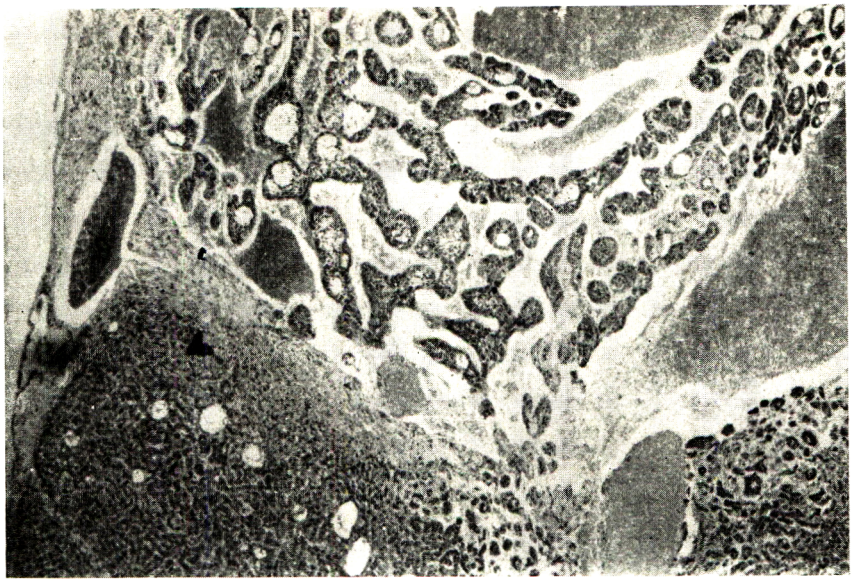


FIG. 13. Type (b) liver tumour from mouse fed 100 ppm DDT. Haematoxylin and eosin ×132.

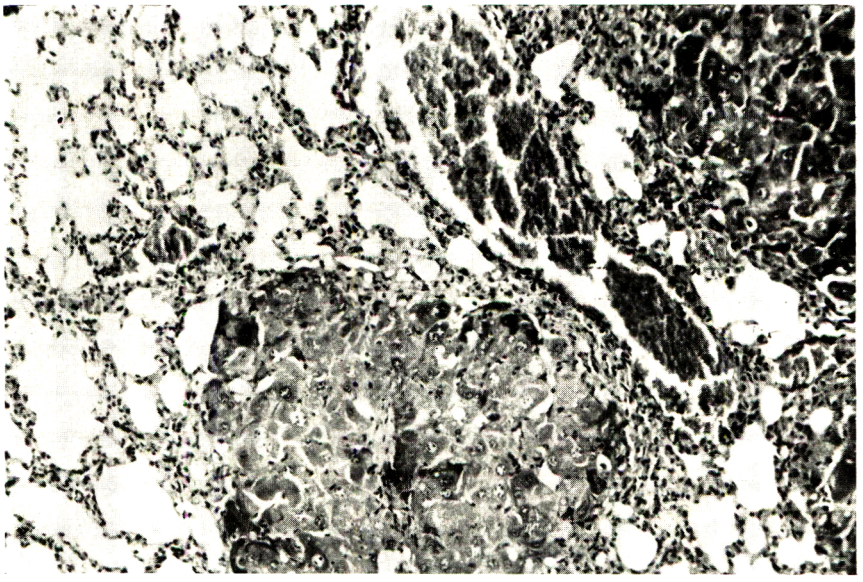


FIG. 14. Multiple metastases of the hepatoma shown in Fig. 13. Haematoxylin and eosin ×530.

included hydropic change, fatty change and hyaline-droplet formation. Focal necrosis was also found. The vast majority of these tumours were found in livers that also showed type (a) lesions. In many cases the two types of lesion were contiguous. These liver tumours with the papilliform and adenoid growth pattern are indicated as type (b) in the various tables to follow.

Mice with type (b) tumours often had type (a) growths as well, but are not included in the (a) column in the tables summarizing tumour incidence.

CF1 mice fed diets containing dieldrin or DDT showed structural changes in the liver parenchymal cells corresponding to those described for DDT by Fitzhugh & Nelson (1947). Enlargement of the centrilobular liver cells was seen with a trend toward margination of the cytoplasmic chromoidal material, and an increase in nuclear size and basophilia (Fig. 8). With longer exposure some centrilobular fatty change occurred and more liver cells in the hepatic lobule showed the nuclear changes and the development of increased cytoplasmic eosinophilia with loss of internal detail. In tumours of liver parenchymal cells in mice fed 10 ppm dieldrin, many of the cells in the mass showed these latter changes. Some liver tumour cells in animals fed dieldrin showed an unusual form of lipid vacuolation in which mnltiple fine droplets were present without nuclear displacement. Necrosis of individual or groups of cells was also found within the growth.

Study 1. An increased incidence of liver tumours was present at all dietary concentrations of dieldrin (Table 1), the increase being greatest at 10 ppm.

ADAB-treated females had a high incidence of liver tumours. Some of the livers in these mice showed areas of fibroplasia and bile-duct proliferation, but the hepatic-cell tumours all showed consistent changes allowing them to be classified into type (a) or (b). Two of the female ADAB-treated mice with type (b) growths had emboli of liver-tumour cells in the lungs. In the hepatic tumours, very large cells with karyomegaly and numerous mitoses were frequently found. No evidence of metastasis of these liver tumours to abdominal lymph nodes was found in the available material.

The group fed 10 ppm dieldrin showed a high incidence of hepatic growths. The proportion of livers showing the less well differentiated, type (b), growths exceeded the simpler, type (a), tumours in both male and female mice. Some animals had sequestered and almost wholly necrotic liver growths in the peritoneal cavity, often surrounded by omentum.

Detection of secondary tumour growth in the lung was difficult, as serial sections through a lung with small intravascular emboli commonly showed only a few positive slides. Two male control mice with type (b) lesions had small pulmonary intravascular emboli of cells (Figs 9 & 10), as had the various dieldrin-fed animals indicated in Table 1.

In mice killed or dying after 21 months on diets containing 0·1 or 1·0 ppm dieldrin, some cells in the liver showed enlargement and the morphological changes consistent with exposure of the animals to organochlorine compounds.

Experiment 2.1. Dose–response relationship. In groups given 10 or 20 ppm dieldrin the numbers of animals available for examination were much reduced because of early losses from acute toxicity and also because of removal of some animals from the experiment for other studies (Table 2). A male and a female mouse fed 2·5 ppm dieldrin had type (b) tumours with metastatic tumour deposits in the lungs (Figs 11 & 12). The lung lesions comprised massive secondary tumours and small emboli of tumour cells in pulmonary vessels.

Experiment 2.2. Diet sterilization. A rather high incidence of simple nodular type (a) growths was found in the control animals in these experiments (Table 3). One control male

Table 1. *Incidence of tumours in mice fed dieldrin for 132 wk or DAB for 26 wk (Study 1)*

Dietary concn (ppm)	No. of animals	Percentage of animals with tumours of									
		Liver				Lung					
		Type*			With secondary tumour deposits in lung‡			Lymphoid tissue	Kidney	Testes/ ovaries	Other tissues
		(a)	(b)	Total†		Adenoma	Carcinoma				
Dieldrin											
Male											
0	288	16	4	20 (16–25)	0·7 (2)	33	8	35	9	2	6
0·1	124	22	4	26 (18–35)	0·8 (1)	38	11	21	12	2	3
1·0	111	23	8	31 (23–41)	0·9 (1)	38	12	20	8	—	5
10·0	176	37	57	94 (89–97)	0·6 (1)	18	1	24	1	—	2
Female											
0	297	13	—	13 (9–17)	—	16	6	40	1	15	7
0·1	90	23	4	27 (18–38)	—	26	13	50	1	12	9
1·0	87	31	6	37 (26–48)	1·1 (1)	34	14	54	—	19	17
10·0	148	37	55	92 (86–96)	4·1 (5)	10	0	5	—	1	1
ADAB											
Male											
600	23	13	4	17 (5–38)	—	39	0	9	—	—	61§
Female											
600	21	43	38	81 (58–94)	9·5 (2)	33	0	5	—	—	—

ADAB = 4-Amino-2,3-dimethylazobenzene

*(a) Simple nodular growth of parenchymal cells; (b) areas of papilliform and adenoid growth of tumour cells.

†With 95% fiducial limits based on binomial distribution as given in Biometrika, Tables for Statisticians, **1,** Table 41.

‡Figures in parenthesis are the actual number of mice showing this lesion.

§Comprising haemangiosarcomas and anaplastic sarcomas.

Table 2. *Incidence of tumours in mice fed dieldrin for 128 wk (Experiment 2.1: Dose–response relationship)*

Dietary concn of dieldrin (ppm)	No. of animals	Percentage of animals with tumours of									
		Liver				Lung					
		Type*									
		(a)	(b)	Total†	With secondary tumour deposits in lung‡	Adenoma	Carcinoma	Lymphoid tissue	Kidney	Testes/ ovaries	Other tissues
					Males						
0	78	12	—	12 (6–21)	—	58	1	28	14	0	13
1·25	30	13	7	20 (7–38)	—	57	3	27	13	0	7
2·5	30	40	3	43 (25–63)	2 (1)	37	3	17	13	0	7
5·0	30	77	10	87 (69–97)	—	47	3	13	0	0	7
10·0	11	36	9	45 (16–77)	—	18	0	0	9	0	0
20·0	17	18	53	71 (44–90)	—	6	0	0	0	0	6
					Females						
0	78	10	—	10 (4–19)	—	31	10	28	0	26	14
1·25	30	17	—	17 (6–35)	—	23	0	20	0	40	13
2·5	28	39	4	43 (22–63)	4 (1)	11	0	18	0	14	11
5·0	30	43	17	60 (41–77)	—	10	3	3	0	10	7
10·0	17	41	12	53 (28–77)	—	6	0	18	0	6	0
20·0	21	24	14	38 (17–62)	—	0	0	0	0	0	0

*(a) Simple nodular growth of parenchymal cells; (b) areas of papilliform and adenoid growth of tumour cells.
†With 95% fiducial limits based on binomial distribution as given in Biometrika, Tables for Statisticians, **1**, Table 41.
‡Figures in parenthesis are the actual number of mice showing this lesion.

Table 3. *Incidence of liver tumours in mice fed dieldrin for 2 yr (Experiment 2.2: Diet sterilization)*

Dietary concn of dieldrin (ppm)	No. of animals	Percentage of animals with tumours of									
		Liver				Lung					
		Type*			With secondary tumour deposits in lung‡			Lymphoid tissue	Kidney	Testes/ ovaries	Other tissues
		(a)	(b)	Total†		Adenoma	Carcinoma				
Unsterilized diet											
Male											
0	23	30	4	34 (16–56)	4·0 (1)	30	0	13	4	0	9
10	24	58	25	83 (62–95)	—	17	0	4	4	0	4
Female											
0	22	23	—	23 (8–46)	—	32	0	32	0	23	5
10	22	36	23	59 (37–82)	4·0 (1)	9	0	5	0	0	5
Irradiated diet											
Male											
0	30	20	3	23 (10–43)	—	43	3	37	13	0	3
10	10	40	20	60 (26–88)	—	10	0	10	0	0	0
Female											
0	28	11	—	11 (3–29)	—	18	0	32	0	25	10
10	19	32	11	43 (21–68)	—	0	0	0	0	5	0
Irradiated diet and bedding											
Male											
0	24	42	—	42 (23–64)	—	46	4	13	4	0	13
10	22	63	23	86 (64–96)	—	36	0	5	9	0	5
Female											
0	24	17	—	17 (5–37)	—	17	13	2	0	29	17
10	24	42	21	63 (42–82)	—	29	0	21	0	17	0

*(a) Simple nodular growth of parenchymal cells; (b) areas of papilliform and adenoid growth of tumour cells.
†With 95% fiducial limits based on binomial distribution as given in Biometrika, Tables for Statisticians, **1**, Table 41.
‡Figures in parenthesis are the actual number of mice showing this lesion.

given unsterilized diet had a type (b) liver tumour and small emboli of tumour cells were found in the pulmonary vessels. The architecture of this tumour was similar to that in the animals exposed to organochlorine compounds in the diet. The tumour cells were smaller, i.e. nearer to normal size, and did not show the degree of nuclear enlargement or increased basophilia seen in the tumours in the mice fed the higher levels of dieldrin. The occurrence of necrosis was much more limited, while a form of fine lipid vacuolation of the cell cytoplasm common in tumour cells in animals exposed to dieldrin was not seen. One female mouse fed 10 ppm dieldrin in unsterilized diet showed a similar pattern of liver and lung involvement, with large cells in the liver mass showing chlorinated hydrocarbon effects.

Experiment 2.3. DDT and dieldrin. The range of morphological changes in the intact lobules and tumours in the livers of the mice in this experiment was similar to that seen in the other experiments. The same classification was therefore applied. One female mouse fed 100 ppm DDT and one fed 5/50 ppm dieldrin/DDT showed type (b) liver tumours with metastases to the lungs (Figs 13 & 14). The incidence of liver growths was highest in the groups fed the mixture of DDT and dieldrin (Table 4).

Experiment 2.4. Limited exposure. Among the mice killed at the end of the 64-wk exposure period, one control female and one male and one female fed 10 ppm dieldrin had type (a) tumours in their livers. Almost all the 10 ppm animals fed dieldrin for 64 wk and maintained for a further period had liver tumours at 104 wk (Table 5). The size and appearance of the cells within the growths of both types varied considerably. In some cases the microscopic appearance of the cells within the tumours was indistinguishable from that seen in animals still receiving the compound in subsequent experiments; in others the cell size and morphology more closely resembled that in control mice. No evidence of liver tumour metastases was detected in the lung sections of these mice.

Pathology of other tissues

The results summarized above show that all the materials tested increased the incidence of liver tumours at certain dietary concentrations, but in the dieldrin groups in Study 1 and the four experiments of Study 2, no increase was found in the tumour incidence in other tissues (Tables 1–5) and the latent period of tumour induction was not shortened*. ADAB was the only compound associated with an increased incidence of other types of tumour (Table 1). Males receiving ADAB had a 61 % incidence of haemangiosarcomas and anaplastic sarcomas in the subcutaneous tissues and abdominal lymph nodes. In animals receiving 10 ppm dieldrin, the apparent decrease in the incidence of certain tumours was probably associated with a shortened survival time (Tables 1–5).

DISCUSSION

The experiments we have carried out in rats and dogs (Walker *et al.* 1969) and mice support the findings of previous workers who have failed to demonstrate any tumorigenic activity of dieldrin in species other than the mouse. Indeed, Deichmann & MacDonald (1971) found the overall tumour incidence in rats to be significantly lower in treated than in control animals.

The sensitivity of the CF1 mouse to the positive control, ADAB, was established. Males had a high incidence of sarcomas, while in females the livers showed areas of fibroplasia

*Tables giving the tumour incidence of all experimental groups may be obtained from the authors.

Table 4. *Incidence of liver tumours in mice fed DDT or dieldrin/DDT mixture for 2 yr (Experiment 2.3; DDT and dieldrin)*

Dietary concn (ppm)	No. of animals	Percentage of animals with tumours of									
		Liver				Lung					
		Type*			With secondary tumour deposits in lung‡			Lymphoid tissue	Kidney	Testes/ ovaries	Other tissues
		(a)	(b)	Total†		Adenoma	Carcinoma				
Males											
0	47	13	—	13 (5–26)	—	38	0	21	15	0	6
50 DDT	32	28	9	37 (21–56)	—	41	0	16	13	0	3
100 DDT	32	44	9	53 (34–72)	—	50	0	22	16	6	6
5 Dieldrin/50 DDT	32	38	50	88 (72–97)	—	34	3	6	6	3	6
Females											
0	47	17	—	17 (7–31)	—	40	6	36	15	30	19
50 DDT	30	43	7	50 (32–68)	—	20	17	43	7	20	13
100 DDT	32	63	13	76 (57–89)	3·0 (1)	22	3	12	6	19	19
5 Dieldrin/50 DDT	32	28	50	78 (60–91)	3·0 (1)	28	6	13	3	13	0

*(a) Simple nodular growth of parenchymal cells; (b) areas of papilliform and adenoid growth of tumour cells.
†With 95% fiducial limits based on binomial distribution as given in Biometrika, Tables for Statisticians, **1**, Table 41.
‡Figures in parenthesis are the actual number of mice showing this lesion.

Table 5. *Incidence of tumours in mice fed 10 ppm dieldrin for up to 64 wk and surviving for 104 wk (Experiment 2.4: Limited exposure)*

Duration of feeding (wk)	Total no. of animals	No. of animals with tumours of							
		Liver							
		Type*							
		(a)	(b)	Total	Lung	Spleen/lymphatic tissue	Kidney	Testes/ovaries	Other tissues
					Males				
0	18	2	—	2	8	—	4	1	—
2	13	2	—	2	6	2	1	—	—
4	10	—	1	1	3	1	—	—	—
8	10	3	1	4	6	3	2	—	—
16	11	4	—	4	6	—	—	—	1
32	10	4	—	4	5	1	—	—	—
64	13	6	7	13	7	2	1	—	—
					Females				
0	16	1	—	1	4	2	—	4	2
2	9	2	—	2	7	1	—	3	2
4	12	3	1	4	2	1	—	3	1
8	12	4	—	4	6	2	—	4	—
16	8	3	—	3	3	—	—	2	—
32	10	4	—	4	4	3	—	3	—
64	9	6	2	8	2	1	—	3	2

*(a) Simple nodular growth of parenchymal cells; (b) areas of papilliform and adenoid of growth of tumour cells.

and bile-duct proliferation, which were not evident in the untreated controls or in animals receiving DDT or dieldrin.

The cellular hepatic changes produced in mice by DDT and by dieldrin were similar to those already reported in rats by Fitzhugh & Nelson (1947). In particular, an increase in the size of the hepatocytes with cytoplasmic changes were seen, especially in the centrilobular region. These morphological changes regress slowly and to a variable extent after dieldrin exposure is stopped. The concentration of dieldrin in liver decreases only slowly after the cessation of exposure so that a rapid return to a normal histological appearance would not be expected.

The response of the mouse liver to dieldrin may vary between strains. In the present study, the increase in incidence of liver tumours in treated animals over that in controls was higher than that obtained by Davis & Fitzhugh (1962), while Keplinger *et al.* (1968) observed little or no response.

The response of the mouse to dieldrin and a possible interaction with the 2-chloroethanol residue known to be present in food and bedding after sterilization with ethylene oxide was studied. When the effects of diet sterilized with ethylene oxide were compared with those of both irradiated and unsterilized diets, the results indicated that ethylene oxide sterilization had no effect on the response of the mouse to dieldrin.

Oser (1962) and Weinbren (1966) have discussed the difficulties of interpreting data derived from animal experimentation on the carcinogenic effects of chemicals. Oser (1962) also indicated that a lack of agreement exists on the most suitable experimental method to be used. Barnes (1966) considered that studies are required on the mode or modes of action of chemical carcinogens before the hazard from pesticides can be assessed.

In studies carried out at this laboratory, Wright, Potter, Wooder, Donninger & Greenland (1971) examined the effect of dieldrin and phenobarbitone on the subcellular structure of the livers of several species. They found that the response of the smooth endoplasmic reticulum to dieldrin was not so pronounced in the mouse as in the dog and rat. In addition, the observations of Baldwin, Robinson & Parkes (1972) are of particular interest, for they showed that the pretreatment of rats with dieldrin caused an increase in the proportion of the amount excreted as the urinary metabolite, pentachloroketone, while no effect on the pattern of urinary metabolites was seen in mice given a similar pretreatment.

These observations support the comment of Fouts (1970) that mice may be an unsatisfactory species in which to study compounds that induce hepatic microsomal enzyme. While DDT has been shown to induce hepatic microsomal enzymes in the rat, this compound had only minimal effects in mice (Conney, 1967; Cram & Fouts, 1967; Hart & Fouts, 1965). With those few inducers (e.g. phenobarbitone and DDT) that have been studied in man, mouse and rat, man responded more like the rat than the mouse (Fouts, 1970).

Acknowledgements—We wish to express our thanks to Mr. R. O. Rees of the Animal Experimental Unit, Tunstall Laboratory, and Mr. J. W. Barrett of the Statistics Unit, Woodstock Agricultural Research Centre, for their assistance.

REFERENCES

Annau, E. (1953). Estimation of pentose nucleic acid and desoxypentose nucleic acid in the liver and brain tissue of mice following the feeding of the insecticide aldrin. *Can. J. Biochem. Physiol.* **32,** 178.

Baldwin, M. K., Robinson, J. & Parkes, D. V. (1972). A comparison of the metabolism of HEOD (dieldrin) in the CF1 mouse with that in the CFE rat. *Fd Cosmet. Toxicol.* 1972, **10,** 333.

Barnes, J. M. (1966). Carcinogenic hazards from pesticide residues. *Residue Rev.* **13,** 69.

Charles, R. T., Stevenson, D. E. & Walker, A. I. T. (1965). The sterilization of laboratory animal diet by ethylene oxide. *Lab. Anim. Care* **15,** 321.

Conney, A. H. (1967). Pharmacological implications of microsomal enzyme induction. *Pharmac. Rev.* **19,** 317.
Cram, R. L. & Fouts, J. R. (1967). The influence of DDT and γ-chlordane on the metabolism of hexobartial and zoxazoalamine in two mouse strains. *Biochem. Pharmac.* **16,** 1001.
Davis, K. J. & Fitzhugh, O. G. (1962). Tumorigenic potential of aldrin and dieldrin for mice. *Toxic. appl. Pharmac.* **4,** 187.
Deichmann, W. B. & MacDonald, W. E. (1971). Organochlorine pesticides and human health. *Fd Cosmet. Toxicol.* **9,** 91.
Fitzhugh, O. G. & Nelson, A. A. (1947). The chronic oral toxicity of DDT [2,2-bis (p-chlorophenyl-1,1,1-trichloroethane)]. *J. Pharmac. exp. Ther.* **89,** 18.
Fouts, J. R. (1970). The stimulation and inhibition of hepatic microsomal drug-metabolizing enzymes with special reference to effects of environmental contaminants. *Toxic. appl. Pharmac.* **17,** 804.
Hart, L. G. & Fouts, J. R. (1965). Further studies on the stimulation of hepatic microsomal drug metabolizing enzymes by DDT and its analogs. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **249,** 486.
Hodge, H. C., Boyce, A. M., Deichmann, W. B. & Kraybill, H. F. (1967). Toxicology and no-effect levels of aldrin and dieldrin. *Toxic. appl. Pharmac.* **10,** 613.
Jager, K. W. (1970). *Aldrin, Dieldrin, Endrin, and Telodrin. An Epidemiological and Toxicological Study of Long-term Occupational Exposure.* Elsevier Publishing Co., Amsterdam.
Keplinger, M. L., Deichman, Wm. B. & Sala, F. (1968). Effects of combinations of pesticides on reproduction in mice. *Ind. Med. Surg.* **37,** 525.
Oser, B. L. (1962). The experimental induction of cancer by pesticide residues and food additives: its rationale and interpretation. *Residue Rev.* **1,** 1.
Salaman, M. H. (1967). Virus-induced lymphoma in mice. In *Pathology of Laboratory Rats and Mice.* Edited by E. Cotchin and A. J. C. Roe. p. 614. Blackwells Scientific Publications, Oxford.
Song, J. & Harville, W. E. (1964). Carcinogenicity of aldrin and dieldrin in mouse and rat liver. *Fedn Proc. Fedn Am. Socs exp. Biol.* **23,** 336.
Walker, A. I. T. & Poppleton, W. R. A. (1967). The establishment of a specific-pathogen-free (SPF) rat and mouse breeding unit. *Lab. Anim,* **1,** 1.
Walker, A. I. T., Stevenson, D. E., Robinson, J., Thorpe, E. & Roberts, M. (1969). The toxicology and pharmacodynamics of dieldrin (HEOD): Two-year oral exposures of rats and dogs. *Toxic. appl. Pharmac.* **15,** 345.
Weinbren, K. (1966). The liver. In *Systemic Pathology.* Vol. 1. Edited by G. Payling Wright and W. St. Clair Symmens. p. 668. Longmans Green & Co., London.
Wright, A. S., Potter, D., Wooder, M. F., Donninger, C. & Greenland, R. D. (1971). The effects of dieldrin on the subcellular structure and function of mammalian liver cells. *Fd Cosmet. Toxicol.* 1972, **10,** 311.

Toxicologie du dieldrin (HEOD). I. Etude de la toxicité orale à long terme chez la souris

Résumé—L'article décrit deux études comportant cinq épreuves de toxicité orale à long terme et au cours desquelles on a soumis des souris CF1 à des régimes contenant différents taux de dieldrin (pureté >99% HEOD), de *p,p'*-DDT (pureté >99,5%) et d'une combinaison de ces deux produits. On a étudié également l'interaction entre la méthode de stérilisation des aliments et l'apparition de lésions du foie, ainsi que le comportement de ces lésions après la réinstauration du régime initial. On a constaté chez un groupe de témoins positifs, qui recevaient 600 ppm de 4-amino-2,3-diméthylazobenzène (ADAB), que 61% des mâles développaient des sarcomes, mais que les lésions prédominantes chez les femelles étaient des altérations pathologiques du foie. La consommation de 10 ppm ou moins de dieldrin et celle de 100 ppm ou moins de DDT n'a provoqué aucun symptôme de mauvaise santé.

Chez les souris qui recevaient du dieldrin, on a constaté une hypertrophie du foie après 9 mois de régime à 10 ppm, après 19 mois de régime à 5 ppm et après 23 mois de régime à 2,5 ppm; cette hypertrophie est allée de pair avec une baisse du taux de survie dans les trois groupes. Elle n'a pas été décelée cliniquement et le taux de survie n'a pas été affecté chez les animaux qui consommaient 1,25 et 1 ppm de dieldrin. En ce qui concerne le DDT, une hypertrophie du foie et une augmentation de la morbidité ont été observées après 16 mois du régime à 100 ppm. Dans le groupe soumis au régime à 50 ppm, l'hypertrophie s'est manifestée après 24 mois, mais la morbidité n'a pas été modifiée. La méthode de stérilisation des aliments n'a pas influé sur le développement des lésions du foie.

Les lésions du foie observées dans tous les groupes, le groupe témoin compris, ont été classées en types a, simple accroissement nodulaire de cellules du parenchyme du foie, et en types b, zones de croissance papilliforme et adénoïde de cellules tumorales, parfois accompagnées, chez les animaux témoins comme chez les animaux traités, de métastases pulmonaires. Les deux types de lésions étaient concomitants chez de nombreux animaux. L'augmentation de la fréquence des lésions du foie dans les groupes traités était évidente. La fréquence d'autres tumeurs a été plus grande chez les souris traitées à l'ADAB, mais non dans les groupes traités au dieldrin ou au DDT.

Die Toxikologie von Dieldrin (HEOD). I. Langzeitige orale Toxizitätsuntersuchungen an Mäusen

Zusammenfassung—Es wurden zwei Untersuchungen abgeschlossen, die fünf langzeitige orale Toxizitätsversuche umfassten, in denen Dieldrin (Reinheit >99% HEOD), *p,p'*-DDT (Reinheit >99,5%) und eine Kombination dieser zwei Verbindungen an CF1-Mäuse in verschiedenen Konzentrationen verfüttert wurden. Die Wechselwirkung zwischen der Methode der Futtersterilisation und der Entwicklung von Leberveränderungen und das Verhalten der Leberläsion nach der Rückkehr zum Grundfutter wurden ebenfalls untersucht. In einer positiven Kontrollgruppe, die 600 ppm 4-amino-2,3-dimethylazobenzol (ADAB) im Futter erhielt, entwickelten sich bei 61% der männlichen Tiere Sarkome, während pathologische Veränderungen in der Leber die vorherrschenden Läsionen bei den weiblichen Tieren waren. Es wurden keine Krankheitszeichen bei der Verfütterung von Dieldrin in Konzentrationen von 10 ppm oder darunter oder bei der von DDT in Konzentrationen von 100 ppm oder darunter beobachtet.

Bei Mäusen, die im Futter Dieldrin erhielten, war Lebervergrösserung nach 9 Monaten Verfütterung von 10 ppm, nach 19 Monaten Verfütterung von 5 ppm und nach 23 Monaten Verfütterung von 2,5 ppm feststellbar und in allen drei Gruppen mit einer Abnahme der Überlebendenzahl verbunden. Bei den Dosierungen 1,25 und 1 ppm Dieldrin wurde klinisch eine Vergrösserung festgestellt und das Überleben war nicht beeinflusst. Bei DDT wurde Lebervergrösserung in der 100-ppm-Gruppe nach 16 Monaten festgestellt, und die Sterblichkeit war erhöht. In der 50-ppm-Gruppe trat die Vergrösserung nach 24 Monaten in Erscheinung, aber die Sterblichkeit war nicht beeinflusst. Die Methode der Futtersterilisation beeinflusste die Entwicklung von Leberläsionen nicht.

Die in allen Gruppen einschliesslich der Kontrollgruppen beobachteten Leberläsionen wurden als Typen (a), ein einfaches nodulares Wachstum von Leberparenchymzellen, und (b), stellenweises papilliformes und adenoides Wachstum von Tumorzellen, manchmal bei Kontroll- und Versuchstieren von Lungenmetastasen begleitet, klassifiziert. Bei vielen Tieren traten die beiden Läsionstypen gleichzeitig auf. Eine erhöhte Häufigkeit von Leberläsionen wurde in den behandelten Gruppen konstatiert. Die Häufigkeit anderer Tumoren war bei ADAB-behandelten Mäusen erhöht, jedoch nicht in den Dieldrin- und DDT-Gruppen.

Fd Cosmet. Toxicol. Vol. 11, pp. 433–442. Pergamon Press 1973. Printed in Great Britain

The Toxicology of Dieldrin (HEOD*). II. Comparative Long-term Oral Toxicity Studies in Mice with Dieldrin, DDT, Phenobarbitone, β-BHC and γ-BHC

E. THORPE and A. I. T. WALKER

Shell Research Limited, Tunstall Laboratory, Sittingbourne, Kent, England

(*Received 1 January 1973*)

Abstract—A 2-yr oral toxicity study has been completed in which dieldrin (purity > 99% HEOD), *p,p'*-DDT (purity > 99·5%), β-BHC (purity > 99%), γ-BHC (purity > 99·5%) and phenobaritone (purity not less than 98%) were fed to CF1 mice at different dietary concentrations. The experiment was designed to study the development of liver changes in CF1 mice fed compounds known to cause hepatic microsomal-enzyme induction.

Liver enlargement was detected after 50 wk in both sexes given dieldrin (10 ppm) or γ-BHC (400 ppm) and in females fed DDT (100 ppm) or β-BHC (200 ppm), after 60 wk in males given β-BHC (200 ppm) and after 68 wk in males given DDT (100 ppm) and in males and females given phenobarbitone (500 ppm). Liver lesions observed in all groups were classified as hyperplastic foci, type (a) a simple nodular growth of parenchymal cells and type (b) with areas of papilliform and adenoid growth of tumour cells, sometimes associated with lung metastases.

The problem of the classification and diagnosis of type (a) and (b) growths is considered. In addition, the biological significance of the induction of liver tumours in mice and the importance of liver cell hyperplasia are discussed.

INTRODUCTION

In a series of experiments carried out in this laboratory with mice given 10 ppm dieldrin or 100 ppm DDT in the diet, the animals developed enlarged livers and subsequently hepatic tumours after 9 months on the dieldrin diet and after 16 months on the DDT diet (Walker, Thorpe & Stevenson, 1973). In another study carried out to investigate the effect of dieldrin and phenobarbitone on the subcellular structures of the livers of several species (Wright, Potter, Wooder, Donninger & Greenland, 1972), mice fed phenobarbitone for 6 months developed liver enlargement with cellular changes similar to those found with dieldrin and DDT (Stevenson & Walker, 1969).

The development of liver tumours in mice after prolonged exposure to dieldrin was the only long-term adverse effect of the compound in this species, and no changes in the incidence of tumours were found in rats and dogs (Walker, Stevenson, Robinson, Thorpe & Roberts, 1969) or monkeys (Wright *et al.* 1972). As a result of these findings in mice, a further study was initiated to investigate the response of Carworth Farm no. 1 (CF1) mice to various chemicals capable of inducing microsomal enzymes in mammalian liver cells. The compounds selected for the study were dieldrin, DDT, sodium phenobarbitone, β-hexachlorocyclohexane (β-BHC), γ-hexachlorocyclohexane (γ-BHC) and butylated hydroxytoluene (BHT).

*HEOD (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*exo*-1,4-*endo*-5,8-dimethanonaphthalene) is the major constituent of dieldrin.

EXPERIMENTAL

Materials. Recrystallized dieldrin (purity > 99% HEOD) was supplied by Woodstock Agricultural Research Centre, Sittingbourne, Kent, *p,p'*-DDT (purity > 99·5%, twice recrystallized from methanol) was prepared at Tunstall Laboratory, Sittingbourne, Kent, β-BHC (purity > 99%), γ-BHC (purity 99·5%) and BHT (purity > 99%) were supplied by Koch-Light Laboratories, Colnbrook, Bucks., and phenobarbitone sodium (purity not less than 98%) was supplied by British Drug House Ltd., London.

Animals. This study used 450 mice of the CF1 strain, bred and maintained under specific-pathogen-free conditions at this laboratory.

Experimental design and observations

Preliminary experiments. A series of 4-wk feeding studies were carried out in order to determine the dietary concentrations to be used for the main experiment. Various dietary concentrations of the different compounds were fed to groups of six males and six females. Three mice of each sex from each treatment group were autopsied. The liver histology was examined for the presence of cellular changes similar to those found after the intake of 10 ppm dieldrin for 4 wk (Walker *et al.* 1973).

Main experiment. As a result of the preliminary experiments, diets containing 10 ppm dieldrin, 100 ppm DDT, 200 ppm β-BHC, 400 ppm γ-BHC, 500 ppm sodium phenobarbitone or 2500 ppm BHT were fed to groups of 30 male and 30 female mice. The control groups comprised 45 animals of each sex. Litters of weanling mice aged 3 wk were earmarked to enable their distribution within the study to be recorded. After 1 wk, the mice were weighed, caged individually, randomly allocated to treatments and given the appropriate treated diet (Diet 86 powder, Scientific Products Farm, Ash, Kent).

The health and behaviour of all the animals were observed daily. In contrast to the earlier series of experiments (Studies 1 and 2; Walker *et al.* 1973), the mice in this experiment (Study 3) were not sent for autopsy when the intra-abdominal mass reached a size considered injurious to health. Mice with such masses were observed closely and killed only when they had become anorexic and clinically affected. At autopsy, a gross dissection was made of each mouse. Tissue blocks of brain, heart, lungs, liver, kidney and testes or ovary and of any macroscopic lesion were fixed in 10% neutral formalin solution, and processed for paraffin sections. Five μm sections were stained with haematoxylin and eosin.

Comparison between each treatment group and control with respect to mortality and tumour incidence were tested for significance using a 2 × 2 contingency table (Finney, Latscha, Bennett & Hsu, 1963).

RESULTS

Clinical observations

During the first 4 days of the study, 16% of the males and 63% of the females given 2500 ppm BHT died. No clinical signs of intoxication were observed, the animals being found dead. In the preliminary experiments, the dietary concentrations of BHT fed were 1000 and 5000 ppm. Deaths occurred at 5000 ppm but not at 1000 ppm BHT, so an intermediate concentration of 2500 ppm was chosen. Because of these very early losses, the groups receiving BHT were removed from the main study. A further study with BHT was initiated and will be reported separately.

During the first 3 months of the experiment, about 12% of the males and 25% of the females given 200 ppm β-BHC and 10% of the males and 20% of the females receiving 400 ppm γ-BHC died. Some β-BHC mice showed signs of ataxia before death. However, throughout the 2-yr study no signs of intoxication were seen in the dieldrin and DDT groups nor in survivors of the β- and γ-BHC groups. When the mice found dead or killed early in the experiment were excluded, the mortality of the various treatment groups was similar to that of the control group (Fig. 1). However, after exposure for 22 months, the mortality of the 10 ppm dieldrin mice was increased (Fig. 1).

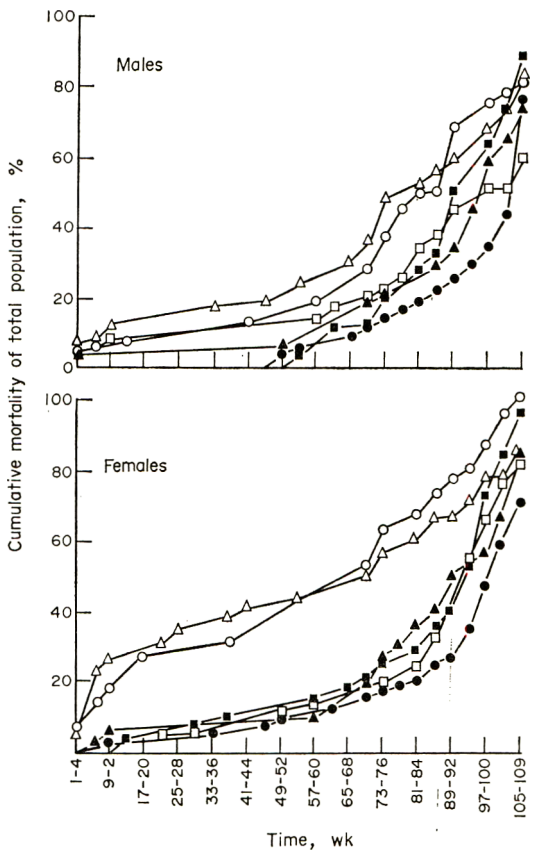


FIG. 1. Cumulative percentage mortality (comprising all mice found dead and those showing signs of ill-health and sent for autopsy) for untreated mice (●) and mice fed 10 ppm dieldrin (■), 100 ppm DDT (▲), 200 ppm β-BHC (△), 400 ppm γ-BHC (○) or 500 ppm sodium phenobarbitone (□).

Liver enlargement was detected by wk 50 in both males and females in the 10 ppm dieldrin and 400 ppm γ-BHC groups and in females in the 100 ppm DDT and 200 ppm β-BHC groups, by wk 60 in males of the 200 ppm β-BHC group, and after wk 68 in males of the 100 ppm DDT group and in both sexes of the 500 ppm phenobarbitone group.

Liver pathology

The gross appearance of the liver lesions was similar in mice given dieldrin, DDT, β-BHC,

γ-BHC or phenobarbitone. The liver was enlarged, with an irregular nodular surface involving one or more lobes. Individual nodules on the surface ranged from a few millimetres to several centimetres in diameter. Many lesions were of normal colour with irregular paler foci; occasional yellow necrotic areas were visible on the surface or on section. No fibrotic induration was detected in the gross specimens. Large vessels were seen below the capsule covering the more extensive nodular growths.

The microscopic changes in these various groups of treated mice were comparable. In a liver lobe with multiple or confluent nodules, it was difficult to separate areas of focal hyperplasia of the parenchyma from some examples of probable liver cell neoplasms. As in the previous studies (Walker *et al.* 1973), if an individual lesion of well-differentiated liver cells showed evidence of involvement of portal-tract elements it was diagnosed as hyperplastic. Because of the similarities of the morphological response to dieldrin, DDT, β- and γ-BHC and phenobarbitone, the arbitrary histological classification of type (a) and type (b) liver-cell tumours described previously (Walker *et al.* 1973) was used for all groups of mice. Hyperplastic foci and type (a) growths were present in the same or other lobes, when type (b) tumours were found. For each mouse, only the most aberrant liver-cell tumour present was recorded. Thus, if a mouse had type (a) and type (b) lesions, only the lesions with the type (b) morphology were recorded in the tabulated results. The incidence of tumours in the various groups of mice is summarized in Tables 1 and 2.

The first liver tumours were found after a 12-month exposure period at autopsy of a male on 10 ppm dieldrin and a female on 400 ppm γ-BHC. In control mice, the earliest liver tumours were found in a male dying at 18 months and a female at 23 months (Table 1). As in the earlier studies, intravascular emboli and metastases of liver tumour cells were found only in the lungs of mice with (b) type tumours. No metastases were found in any other tissues.

The overall incidence of extrahepatic tumours appeared to be reduced in most groups of treated animals (Tables 1 & 2). These data included animals dying or killed before any tumours occurred. In order to exclude these animals and because of the possible influence of life expectancy on tumour incidence, the data were evaluated to determine the cumulative tumour incidence subsequent to the finding of the first tumour in any tissue in each group of mice. Statistically, significant reductions in neoplasms other than of hepatic-cell origin occurred only in male mice exposed to 100 ppm DDT and 200 ppm β-BHC (Table 2).

DISCUSSION

These experiments have confirmed the previously reported finding of an increased incidence of hyperplastic foci and parenchymal cell tumours in the livers of CF1 mice following the prolonged ingestion of dieldrin and DDT (Walker *et al.* 1973). Exposure to phenobarbitone, β-BHC or γ-BHC was associated with an increased incidence of hepatic lesions in this mouse strain, morphologically indistinguishable from those found with dieldrin and DDT.

The apparent increase in lifespan compared with that recorded in the previous study (Walker *et al.* 1973) was related to the change adopted in experimental procedure, in that during the present study, mice were killed only when anorexic and clinically affected and not when the abdominal enlargement associated with hepatomegaly was considered injurious to health, as in the previous studies.

In a recent report (Tomatis, 1972; Tomatis, Turusov, Day & Charles, 1972) a dietary

Table 1. *Mortality and cumulative incidence of tumours of the liver and other tissues in mice fed dieldrin, DDT, phenobarbitone, β-BHC or γ-BHC for 110 wk*

Compound and dietary concn (ppm)	Deaths and percentage of dead animals with tumours at: 0–17 months N†	0–17 months Tumours: Liver (%)	0–17 months Tumours: Other (%)	0–21 months N†	0–21 months Tumours: Liver (%)	0–21 months Tumours: Other (%)	0–25 months N†	0–25 months Tumours: Liver (%)	0–25 months Tumours: Other (%)	End of experiment: 26 months N‡	26 months Tumours: Liver (%)	26 months Tumours: Other (%)	Totals (0–26 months) N	Totals (0–26 months) Tumours: Liver (%)	Totals (0–26 months) Tumours: Other (%)
Males															
Control (0)	6	0	83	11	18	64	25	8	76	20	45	90	45	24	82
Dieldrin (10)	4	100**	0*	11	100**	27	27**	100**	56	3*	100	67	30	100**	57*
DDT (100)	7	57	43	10	70*	60	22	17**	55	8	75	63	30	77**	57*
Phenobarbitone (500)	6	50	33	12	58	42	18	72**	56	12	92	83	30	80**	67
β-BHC (200)	13**	46	15*	17*	59	24	26*	69**	31*	4	100	100	30	73**	40
γ-BHC (400)	7	86**	29	15	93**	47	24	96**	54	5	80	100	29	93**	48**
Females															
Control (0)	4	0	100	11	0	64	30	17	80	14	36	86	44	23	82
Dieldrin (10)	6	50	17*	18*	78**	56	28*	86**	61	2*	100	50	30	87**	60
DDT (100)	7	57	29	14	71**	64	24	83**	63	6	100*	33	30	87**	57*
Phenobarbitone (500)	5	20	80	12	50*	75	24	71**	75	4	100	100	28	75**	79
β-BHC (200)	16**	13	19*	20**	25	35	25	36	44*	5	80	60	30	43	47**
γ-BHC (400)	18**	50	28*	23**	61**	39	28	68**	46*	1*	100	100	29	69**	48**

†No. of animals that died by the month stated.
‡No of animals killed at the end of the experiment.
Values marked with asterisks differ significantly from those of controls: *$P < 0.05$; **$P < 0.01$.

Table 2. *Incidence of tumours in mice fed dieldrin, phenobarbitone or β- or γ-BHC for up to 110 wk*

Compound and dietary concn (ppm)	No. of animals	Percentage of animals with tumours: Liver Type (a)	Liver Type (b)	Liver Total‡	Liver With secondary deposits in lung	Lung	Lymphoid tissue	Kidney	Testes/ ovaries	Other tissues	Percentage incidence of tumours† No. of animals	Liver (a) + (b)	Other tissues
Males													
Control (0)	45	20	4	24 (13–38)	0	60	35	6	2	6	45	24	82
Dieldrin (10)	30	47	53	100 (—)	3	37	10	3	3	3	24	100**	71
DDT (100)	30	47	30	77 (57–90)	0	37	13	3	0	3	29	79**	59*
Phenobarbitone (500)	30	53	27	80 (62–93)	0	50	10	3	0	3	29	83**	69
β-BHC (200)	30	40	33	73 (53–87)	13	27	6	0	3	10	24	92**	50*
γ-BHC (400)	29	38	55	93 (77–99)	10	40	13	3	0	10	28	96*	64
Females													
Control (0)	44	23	0	23 (12–37)	0	61	36	7	2	7	44	23	82
Dieldrin (10)	30	40	47	87 (69–97)	17	26	26	0	7	3	28	93**	64
DDT (100)	30	47	40	87 (69–97)	3	30	20	3	7	3	27	96**	63
Phenobarbitone (500)	28	43	32	75 (55–89)	0	33	25	0	10	25	27	78**	81
β-BHC (200)	30	30	13	43 (25–63)	0	17	20	0	0	13	19	68**	74
γ-BHC (400)	29	34	34	69 (49–85)	3	33	7	0	3	12	21	95**	67

†The percentage incidence of tumours subsequent to the finding of the first tumour in any tissue in each group of mice.
‡With 95% confidence limits based on binomial distribution as given in *Biometrika, Tables for Statisticians* **1**, Table 41.
Values marked with asterisks differ significantly from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

intake of 250 ppm technical DDT was shown to result in an increased incidence of liver tumours in CF1 and BALB/c mice. Nagasaki, Tomii, Mega, Marugami & Ito (1971) found a 100% incidence of liver tumours in male dd mice fed 660 ppm technical BHC.

Nodular liver lesions in mice pose problems in respect of their diagnosis. The term 'hepatoma' is inappropriate to describe these lesions because of different connotations in mouse and human pathology (International Agency for Research on Cancer, 1971). Continued parenchymal cell hyperplasia, benign neoplasms and primary parenchymal cell carcinoma in rodent liver may be a progressive spectrum of change (*British Medical Journal*, 1972; Popper & Schaffner, 1957), although proof of this kind of evolution is lacking. Spontaneous liver nodules are not uncommon in adult males of certain mouse strains and the incidence of lesions can be increased by exposure of neonates or adults to various chemicals (Warwick, 1971). Morphology alone is inadequate to identify hyperplastic lesions from some liver cell neoplasms in mice. Even the use of techniques to investigate the biological characteristics of such lesions is open to debate, for example nodules have been successfully transplanted when there was no evidence of invasion or metastasis in the donor animal (Andervont & Dunn, 1952; Edwards, Dalton & Andervont, 1942).

To place our data on CF1 mice in perspective against this background, the type (a) growths should probably be classified as hyperplastic nodules, since we have no evidence of local invasion, metastases or transplantability to support a diagnosis of neoplasia. The nodules classified as type (b) on morphology are to be regarded as neoplasms, since metastases and successful transplantation into random-bred adult mice have been established for example for this lesion derived from dieldrin- or phenobarbitone-treated mice (E. Thorpe and R. O. Rees, to be published, 1973). On these criteria, the prolonged ingestion of all the compounds studied is associated with an increase in hyperplastic nodules and liver cell tumours in CF1 mice of both sexes. No metastases were detected in mice fed phenobarbitone in this experiment when liver tumours with (b) type growth patterns were found*.

Despite the massive size and multiplicity of the liver lesions in treated mice in the present experiments only a minimum shortening of the lifespan occurred during the 2-yr experiment, even when the incidence of gross hepatic changes approached 100% and had been present for several months as determined by palpation. However, no increase in neoplasms in non-hepatic tissues was found in any of the treated groups and reduced numbers of tumours occurred in male mice exposed to 100 ppm DDT and 200 ppm β-BHC. The factors leading to fewer tumours in other tissues are unknown.

The biological significance of the altered incidence of liver tumours in mice presents a major problem. The concept that an increased incidence of liver tumours in mice constitutes insufficient evidence to classify a compound as carcinogenic has been proposed recently (Roe & Grant, 1970; Grasso & Crampton, 1972). Support for this view is given by the diversity of factors shown to influence the development of liver cell tumours in mice, notably the protein or calorific value of the diet (Tannenbaum & Silverstone, 1949a,b), the source of the diet (Heston, Vlahakis & Deringer, 1960), the strain of mouse (Bonser, Clayson, Jull & Pyrah, 1952; Keplinger, Deichmann & Sala, 1968; Tomatis, 1971), alterations in the endocrine status (Warwick, 1971) and the microbial flora of the animals (Roe & Grant, 1970).

Liver cell hyperplasia as shown by an increased liver DNA is an early response to dieldrin and phenobarbitone in the mouse but not in the rat or dog (Wright *et al.* 1972). The potential

*In a subsequent experiment in which mice have been exposed to 1000 or 3000 ppm phenobarbitone, lung metastases have been found when (b) type growths were present.

importance of cell division in long-term effects has been shown by studies in which partial hepatectomy and the consequent cell replication enhances the carcinogenicity of some compounds (Chernozemski & Warwick, 1970; Hollander & Bentvelzen, 1968). An increased rate of liver growth after partial hepatectomy was not observed in rats given dieldrin (Gershbein, 1970). In CF1 mice given dieldrin or phenobarbitone the liver cell hyperplasia must be considered as a possible factor in the increased incidence of liver tumours in this strain. CF1 mice, particularly males, have a background incidence of hyperplastic nodules and liver tumours of unknown aetiology. Compounds eliciting a sustained mitotic response in mouse liver could increase the susceptibility of the animals to the unknown oncogenic stimulus responsible for the spontaneous lesions in untreated mice. However, any specific role of hyperplasia or microsomal enzyme induction in the evolution of liver cell tumours in mice has yet to be established.

A critical assessment of the carcinogenic potential to man of a chemical where clearcut variation between species exists can be obtained only from epidemiological studies in man exposed to high levels of the material for long periods. This type of study is possible in the case of workers occupationally exposed to dieldrin (K. M. Jager, to be published 1973) and in long-term patients given phenobarbitone (J. Clemmesen, to be published 1973).

Acknowledgements—We wish to express our thanks to Mr. R. O. Rees of the Animal Experimental Unit, Tunstall Laboratory, and Mr. P. F. Hunt of the Statistics Unit, Sittingbourne Laboratories, for their assistance.

REFERENCES

Andervont, H. B. & Dunn, T. B. (1952). Transplantation of spontaneous and induced hepatomas in inbred mice. *J. natn. Cancer Inst.* **13,** 455.

Bonser, G. M., Clayson, D. B., Jull, J. W. & Pyrah, L. N. (1952). Carcinogenic properties of 2-amino-1-naphthol hydrochloride and its parent amine 2-naphthylamine. *Br. J. Cancer* **6,** 412.

British Medical Journal (1972). Aetiology of liver cancer. *ibid* **1,** 261.

Chernozemski, I. N. & Warwick, G. P. (1970). Liver regeneration and induction of hepatomas in $B6AF_1$ mice by urethan. *Cancer Res.* **30,** 2685.

Edwards, J. E., Dalton, A. J. & Andervont, H. B. (1942). Pathology of a transplantable spontaneous hepatoma in a C3H mouse. *J. natn. Cancer Inst.* **2,** 555.

Finney, D. J., Latscha, R., Bennett, B. M. & Hsu, P. (1963). *Tables for Testing Significance in a 2 × 2 Contingency Table.* Cambridge University Press, Cambridge.

Gershbein, L. L. (1970). Effects of insecticides on rat liver regeneration. *Res. Commun. Chem. Path. Pharmac.* **1,** 740.

Grasso, P. & Crampton, R. F. (1972). The value of the mouse in carcinogenicity testing. *Fd Cosmet. Toxicol.* **10,** 418.

Heston, W. E., Vlahakis, G. & Deringer, Margaret K. (1960). High incidence of spontaneous hepatomas and the increase of this incidence with urethan in C3H, C3Hf, and C3He male mice. *J. natn. Cancer Inst.* **24,** 425.

Hollander, C. F. & Bentvelzen, P. (1968). Enhancement of urethan induction of hepatomas in mice by prior partial hepatectomy. *J. natn. Cancer Inst.* **41,** 1303.

International Agency for Research on Cancer (1971). Report and recommendations of subcommittee on morphology, epidemiology and pathology. In *Liver Cancer.* Proceedings of a Working Conference held at the Chester Beatty Research Institute, London, England, on 30 June to 3 July 1969. IARC Scientific Publications No. 1. p. 173. International Agency for Research on Cancer, Lyon.

Keplinger, M. L., Deichmann, W. B. & Sala, F. (1968). Effects on combinations of pesticides on reproduction in mice. *Ind. Med. Surg.* **37,** 525.

Nagasaki, H., Tomii, S., Mega, T., Marugami, M. & Ito, N. (1971). Development of hepatomas in mice treated with benzene hexachloride. *Gann* **62,** 431.

Popper, H. & Schaffner, F. (1957). *Liver: Structure and Function.* p. 593. McGraw-Hill, New York.

Roe, F. J. C. & Grant, G. A. (1970). Inhibition by germ-free status of development of liver and lung tumours in mice exposed neonatally to 7,12-dimethylbenz(a)-anthracene: Implications in relation to tests for carcinogenicity. *Int. J. Cancer* **6,** 133.

Stevenson, D. E. & Walker, A. I. T. (1969). Hepatic lesions produced in mice by dieldrin and other hepatic enzyme-inducing compounds. *Eur. J. Toxicol.* **2,** 83.

Tannenbaum, A. & Silverstone, H. (1949a). The genesis and growth of tumors. IV. Effects of varying the proportion of protein (casein) in the diet. *Cancer Res.* **9,** 162.

Tannenbaum, A. & Silverstone, H. (1949b). The influence of the degree of calorie restriction in the formation of skin tumors and hepatomas in mice. *Cancer Res.* **9,** 724.

Tomatis, L. (1972). In *International Agency for Research on Cancer Annual Report for 1971.* p. 70. IARC, Lyon.

Tomatis, L., Turusov, V., Day, N. & Charles, R. T. (1972). The effect of long-term exposure to DDT on CF-1 mice. *Int. J. Cancer* **10,** 489.

Walker, A. I. T., Stevenson, D. E., Robinson, J., Thorpe, E. & Roberts, M. (1969). The toxicology and pharmacodynamics of dieldrin (HEOD): Two-year oral exposure of rats and dogs. *Toxic. appl. Pharmac.* **15,** 345.

Walker, A. I. T., Thorpe, E. & Stevenson, D. E. (1973). The toxicology of dieldrin (HEOD). Long-term oral toxicity studies in mice. *Fd Cosmet. Toxicol.* **11,** 415.

Warwick, G. P. (1971). Metabolism of liver carcinogens and other factors influencing liver cancer induction. In *Liver Cancer.* Proceedings of a Working Conference held at the Chester Beatty Research Institute, London, England, on 30 June to 3 July 1969. IARC Scientific Publications No. 1. p. 121. International Agency for Research on Cancer, Lyon.

Wright, A. S., Potter, D., Wooder, M. F., Donninger, C. & Greenland, R. D. (1972). The effects of dieldrin on the subcellular structure and function of mammalian liver cells. *Fd Cosmet. Toxicol.* **10,** 311.

Toxicologie de la dieldrine (HEOD). II. Etudes comparatives de la toxicité orale à long terme, chez la souris, de la dieldrine, du DDT, du phénobarbital, du β-BHC et du γ-BHC

Résumé—Les auteurs ont terminé une étude de toxicité orale, d'une durée de 2 ans, au cours de laquelle ils ont administré à des souris CF1 de la dieldrine (pure à plus de 99%), du *p,p'*-DDT (pur à plus de 99,5%), du β-BHC (pur à plus de 99%), du γ-BHC (pur à 99,5%) et du phénobarbital (pur à au moins 98%) incorporés à différents taux dans le régime alimentaire.

L'essai était conçu de façon à permettre l'étude de l'évolution des altérations du foie chez des souris CF1 auxquelles on faisait consommer des produits connus pour provoquer une induction d'enzymes microsomiques hépatiques.

De l'hypertrophie du foie a été décelée après 50 semaines chez les souris des deux sexes qui avaient reçu de la dieldrine (10 ppm) ou du γ-BHC (400 ppm) et chez les femelles qui avaient consommé du DDT (100 ppm) ou du β-BHC (200 ppm), après 60 semaines chez les mâles qui avaient reçu du β-BHC (200 ppm) et après 68 semaines chez les mâles qui avaient reçu du DDT (100 ppm) et chez les mâles et les femelles qui avaient reçu du phénobarbital (500 ppm). Les lésions du foie observées dans tous les groupes ont été classées en foyers hyperplasiques, en lésions de type (a), simple croissance nodulaire de cellules parenchymales, et lésions de type (b), à zones de croissance papilliforme et adénoïde de cellules tumorales, parfois associées à des métastases pulmonaires.

Les auteurs examinent le problème de la classification et du diagnostic des croissances des types (a) et (b). Ils commentent en outre la signification biologique de l'induction de tumeurs du foie chez la souris et l'importance de l'hyperplasie des cellules hépatiques.

Die Toxikologie von Dieldrin (HEOD). II. Vergleichende langzeitige orale Toxizitätsuntersuchungen an Mäusen mit Dieldrin, DDT, Phenobarbital, β-BHC und γ-BHC

Zusammenfassung—Eine zweijährige orale Toxizitätsuntersuchung wurde abgeschlossen, bei der Dieldrin (Reinheit >99% HEOD), *p,p'*-DDT (Reinheit >99,5%), β-BHC (Reinheit >99%), γ-BHC (Reinheit >99,5%) und Phenobarbital (Reinheit nicht unter 98%) an CF1-Mäuse in verschiedenen Konzentrationen im Futter verfüttert wurden. Der Versuch hatte den Zweck, die Entwicklung von Änderungen in der Leber von CF1-Mäusen zu untersuchen, an die Verbindungen verfüttert wurden, deren induktionsauslösende Wirkung gegen mikrosomale Leberenzyme bekannt war.

Lebervergrösserung wurde nach 50 Wochen bei beiden Geschlechtern entdeckt, die Dieldrin (10 ppm) oder γ-BHC (400 ppm) erhielten, ferner bei weiblichen Tieren, an die DDT (100 ppm) oder β-BHC (200 ppm) verfüttert wurde, nach 60 Wochen bei männlichen, die β-BHC (200 ppm) erhielten, nach 68 Wochen bei männlichen Tieren, die DDT (100 ppm), und bei männlichen und weiblichen, die Phenobarbital (500 ppm) erhielten. Die bei allen Gruppen beobachteten Leberläsionen wurden als hyperplastische Knötchen, Type (a) einfaches nodulares Wachstum von Parenchymzellen, und Type (b) mit Stellen papilliformen und adenoiden Wachstums von Tumorzellen, manchmal verbunden mit Lungenmetastasen, klassifiziert.

Das Problem der Klassifikation und Diagnose des Wachstums der Typen (a) and (b) wird besprochen. Ausserdem wird die biologische Bedeutung der Induktion von Lebertumoren bei Mäusen und die Bedeutung von Leberzellenhyperplasie erörtert.

Fd Cosmet. Toxicol. Vol. 11, pp. 443–457. Pergamon Press 1973. Printed in Great Britain

Sensitivity of Feeding Tests in Detecting Carcinogenic Properties in Chemicals: Examination of 7,12-Dimethylbenz[*a*]anthracene and Oxidized Linoleate

MARGARET G. CUTLER and R. SCHNEIDER

Department of Clinical Pharmacology, Medical School, Birmingham, England

(*Received 17 October 1972*)

Abstract—The sensitivity of feeding tests in detecting the carcinogenic properties of a small dose of 7,12-dimethylbenz[*a*]anthracene in female rats was increased when the compound was given to rats at an earlier age and when a high-fat diet containing 1% polyoxyethylene monostearate was administered. Such modifications to the feeding regime also enabled the tumorigenic properties of oxidized linoleate to be detected more readily in female rats.

An increased proportion of female rats bore cervical sarcomas and mammary adenocarcinomas after addition of oxidized linoleate to their diet, and in male rats the incidence of interstitial cell tumours of the testis was raised. Feeding of oxidized linoleate to mice resulted in an increase in benign ovarian tumours and of other benign tumours among the females. The possible risk to the population from the autoxidation of lipids is discussed.

INTRODUCTION

The methods in use for testing compounds for carcinogenic activity and the limitations of these methods have been reviewed by Arcos, Argus & Wolf (1968). Minor alterations of procedure can change the sensitivity of these methods for detecting carcinogenic properties in chemicals. For example, a high proportion of fat in the diet can increase the incidence of neoplasms induced in the skin and mammary glands (Gammal, Carroll & Plunkett, 1967; Tannenbaum, 1942) and younger animals have been shown to be more susceptible than adults to the induction of tumours in many organs (Clayson, 1962; Toth, 1968).

In the present experiments, the sensitivity of a normal type of feeding test for detecting the carcinogenic risk of 7,12-dimethylbenz[*a*]anthracene (DMBA) to rats was compared with that of a test in which the diet was modified and the feeding of the carcinogen to the animals was started at an earlier age. The dosage of DMBA selected was that reported to give a low yield of neoplasms with a fairly long latent period (Gruenstein, Meranze, Thatcher & Shimkin, 1966; Huggins, Grand & Brillantes, 1961; Huggins, Morii & Grand, 1961).

Similar experiments were carried out in rats and mice with oxidized linoleic acid. Linoleic acid, a major component of many seed oils (Ralston, 1948), undergoes autoxidation at ambient temperatures to yield hydroperoxides and a wide range of secondary degradation products (Bolland, 1946; Dulog & Burg, 1963; Farmer & Sutton, 1943). Fatty acid hydroperoxides are toxic. They have been shown to inhibit cell division (Franz & Cole, 1962; Stilwell, Maroney & Wilbur, 1959; Wilbur, Wolfson, Kenaston, Ottolenghi, Gaulden & Bernheim, 1957), to be mutagenic to Neurospora and to Aspergillus (Dickey, Cleland & Lotz, 1949; Van Arkel, 1958), to reduce the viscosity of DNA (Fisher & Wilbur, 1954) and to produce epidermal hyperplasia in the skin of the newt, a reaction suggestive of

carcinogenic activity (Arffmann, 1964; Glavind & Arffmann, 1970). The hydroperoxide of linoleic acid was found by Holman & Greenberg (1958) and by Horgan, Philpot, Porter & Roodyn (1957) to be the most toxic of the fatty acid hydroperoxides they tested.

Three dietary levels of oxidized linoleic acid were used in the present experiments. Since oxidized linoleate destroys dietary vitamins, essential fatty acids and proteins, the compound was given for only a few days of each week so as to minimize some of the toxic side effects (Kaunitz, 1953; Rao, 1960).

EXPERIMENTAL

Animals. Altogether, 531 rats of the Birmingham Wistar outbred strain and 350 mice of the CBA strain of the Laboratory Animals Centre, Carshalton, Surrey, were used. Both rats and mice were reared by specific-pathogen-free foster parents and were maintained in cages of not more than five in a barrier-controlled area of the animal house. Temperature, humidity and light were similar for all animals. Animals were examined daily and any abnormalities observed were recorded. Animals that appeared unwell were isolated and those that were moribund, bore palpable tumours or showed a marked decrease in body weight were killed. Autopsies were performed on all animals that died or were killed and histological preparations were made of all tissues that appeared abnormal.

Materials. DMBA was obtained from L. Light & Co. Ltd., Colnbrook, Bucks. Oxidized linoleic acid (OXLIN) was prepared by bubbling oxygen at room temperature for 72 hr through antioxidant-free linoleic acid obtained from Price's Ltd., Bromborough, Cheshire. Ultraviolet absorption analysis of several batches showed the product to contain 29·0 ± 0·9% conjugated diene. The average composition of OXLIN examined by thin-layer chromatography on silica-gel G plates using hexane–ether–acetic acid (60:40:1, by vol.) with iodine vapour as a visualizer, is shown in Table 1. Identification of the compounds was performed according to Freeman (1964). OXLIN was found to be stable for several weeks at −20°C and was stored at that temperature.

Table 1. *Mean composition of batches of OXLIN prepared*

Component	Composition (%)
Linoleic acid hydroperoxide	25·1 ± 1·4
Epoxy acids	0·8 ± 0·6
Keto acids	2·2 ± 0·8
Monocarboxylic acids esterified with hydroxyketo acids	1·5 ± 1·1
Dicarboxylic acids esterified with hydroxyketo acids	2·5 ± 0·7
Dihydroacids esterified with keto acid or dicarboxylic acid	3·1 ± 0·9
Semialdehydes of dicarboxylic acids	0·6 ± 0·4
Dicarboxylic acids	3·0 ± 0·3
Dicarboxylic acids esterified with a mixture of components	6·2 ± 0·9
Polymeric material	4·3 ± 0·7
Linoleic acid	49·4

Values are means ± SEM.

Diets. In the normal type of feeding test, rats and mice were given a diet of Spiller's rat cake, which was found in a typical analysis to contain 5% fat, 21% protein and 57% carbohydrate. A high-fat diet used in the modified feeding test consisted of a mixture of 79% rat-cake powder, 13% cod-liver oil, 7% fish meal and 1% polyoxyethylene monostearate, and contained 16% fat, 24% protein and 39% carbohydrate. The fatty acid composition of the lipids in Spiller's rat cake and in the high-fat diet are shown in Table 2. These were estimated by gas–liquid chromatography of the methyl esters of fatty acids, as described by Tame & Dils (1967).

Table 2. *Fatty acid composition of the lipids in the modified diet and in Spiller's rat cake*

Fatty acid (no. of carbon atoms: no. of double bonds)	Proportion (%) in	
	High-fat diet	Spiller's rat-cake
14:0	8·3	4·4
15:0	0·1	4·4
16:0	14·3	20·9
16:1	1·9	3·2
17:0	0·2	1·9
18:0	3·1	4·6
18:1	14·3	31·4
18:2	48·2	28·0
18:3	4·0	1·1
20	3·0	—
22	2·5	—

Feeding experiments

Rats: Normal feeding regimen. As shown in Table 3, ten groups of rats were used in the normal type of feeding test. The control groups (C) consisted of 50 females and 50 males and received no additional treatment. The DMBA groups consisted of 20 females and 20 males each given 2·5 mg DMBA at the age of 6 wk. OXLIN was administered in the diet at three different dose levels to rats of groups OX1, OX2 and OX3, commencing when the rats were 6 wk old (Table 3). The highest dose of OXLIN that the rats could tolerate without loss of body weight was found to be 10% OXLIN in rat-cake powder fed for 3 consecutive days/wk and this was given to 20 females and 20 males of group OX3. Half of this dose level was given to 20 females and 20 males of group OX2 and a quarter of the highest dose level was given to 25 females and 20 males of group OX1. The amount of the experimental diet consumed by each cage of rats was recorded daily and the administration of OXLIN continued for 56–75 wk until the total doses of OXLIN specified in Table 3 had been reached. These amounts are only a rough guide to the quantity of OXLIN consumed by each animal since no allowance was made for individual variations in food consumption among animals within a cage.

Rats: Modified feeding regimen. Fifty females and 50 males forming the C′ control groups received the high-fat diet and no additional treatment. The 20 female and 20 male rats of group DMBA′ were given the high-fat diet and each received 2·5 mg DMBA at the age of 4 wk. Experimental rats of groups OX1′, OX2′ and OX3′ were bred from parents receiving

Table 3. *General plan of the feeding tests in rats and mice*

Group	Compound administered	Total dosage/animal	No. of animals/group		Length of feeding (wk)	Age when killed (wk)
			Female	Male		
Rats: Normal feeding regimen (Spiller's rat-cake diet)						
C	—	—	50	50	—	110
DMBA	DMBA	2·5 mg	20	20	—	110
OX1	OXLIN	61 ml	25	20	56	110
OX2		122 ml	20	20	56	110
OX3		244 ml	20	20	75	110
Rats: Modified feeding regimen (high-fat diet)						
C′	—	—	50	50	—	110
DMBA′	DMBA	2·5 mg	20	20	—	110
OX1′	OXLIN	61 ml	20	20	56	110
OX2′		122 ml	20	20	56	110
OX3′		244 ml	25	21	73	110
Mice: Normal feeding regimen (Spiller's rat-cake diet)						
C	—	—	21	20	—	80
OX1	OXLIN	12 ml	20	20	52	80
OX3		48 ml	26	21	65	80
Mice: Modified feeding regimen (high-fat diet)						
C′	—	—	50	50	—	100
OX1′	OXLIN	12 ml	20	21	57	100
OX2′		24 ml	20	21	57	100
OX3′		48 ml	20	20	65	100

10% OXLIN in their diet on 1 day/wk. When weaned at 4 wk of age, these animals received OXLIN in their diet at similar dose levels to those employed for rats given the conventional feeding regimen. The high-fat diets containing OXLIN were made from a mixture of 77% rat-cake powder, 5% cod-liver oil, 10% OXLIN, 7% fish meal and 1% polyoxyethylene monostearate. The 25 females and 21 males of group OX3′ received 10% OXLIN in the high-fat diet for 3 days/wk, 20 females and 20 males of group OX2′ were given half of this dose level, and 20 females and 20 males of group OX1′ a quarter of this dose level.

Mice: Normal feeding regimen. Six groups of mice were used in the normal type of feeding test. As shown in Table 3, 21 female and 20 male control (C) animals received a diet of Spiller's rat cake only, 26 females and 21 males in group OX3 received 10% OXLIN in a diet of rat-cake powder for 3 consecutive days/wk from the age of 6 wk, and a quarter of this dose was given to the 20 females and 20 males of group OX1. The amounts of OXLIN consumed by the mice in each cage were recorded daily and feeding was continued for 52–65 wk until the amounts of OXLIN specified in Table 3 had been eaten. All surviving mice were killed at the age of 80 wk.

Mice: Modified feeding regimen. The control groups (C′) consisted of 50 females and 50 males receiving the high-fat diet and no additional treatment (Table 3). Mice of groups OX1′, OX2′ and OX3′ were bred from animals receiving 10% OXLIN in their diet on 1 day/wk. When weaned at 4 wk, 20 females and 20 males of group OX3′ received 10%

OXLIN in the high-fat diet for 3 consecutive days/wk, 20 females and 21 males of group OX2′ received half of this dose of OXLIN, and a quarter of the highest dose was given to 20 females and 21 males in group OX1′. Feeding of OXLIN was continued for 57–65 wk until the total doses specified in Table 3 had been reached. All surviving mice were killed at the age of 100 wk.

Statistical methods

Animals used in the normal type of feeding test were allocated to the various treatment groups by a random number system. For the modified type of feeding test, parent animals were randomly distributed between two groups. The first group of parent animals received OXLIN and high-fat diet and the second group of parent animals were given high-fat diet only. The offspring from the first group of parents were randomly distributed between the OX1′, OX2′ and OX3′ groups of the modified feeding test. Groups C′ and DMBA′ of the modified test were composed of the offspring from the second group of parent animals.

Tumour incidence was assessed by the method described by Arcos *et al.* (1968). The significance of the differences between groups in the incidence of tumours for each specific organ was calculated by the chi-squared test where the numbers in groups were large, and by Fisher's exact treatment of the 2 × 2 tables where small numbers were involved (Fisher, 1954). The significance of differences between groups in the proportion of tumour-bearing animals was assessed by the same procedure. For the evaluation of differences between groups in the age of animals when tumours were detected, White's ranking method and Student's *t* test were used. These comparisons were made only between tumours of similar types.

Results were grouped together only where there were no significant differences between groups in the specific types of tumours. Fisher and Yates' Statistical Tables were used throughout.

RESULTS

Administration of DMBA to rats

The group of females given 2·5 mg DMBA and the modified feeding regimen showed an increased incidence of malignant mammary tumours and malignant tumours in other organs (Table 4). The group of females given a similar dose of DMBA and the normal type of feeding regimen showed no significant increase of malignant tumours, although the incidence of benign plus malignant mammary tumours was raised. Neither feeding regimen demonstrated the carcinogenic properties of this dose of DMBA in males (Table 4), and there was no significant reduction in the latent period of tumour induction in either males or females given 2·5 mg DMBA.

Administration of OXLIN to rats

As shown in Table 5, the proportion of females with benign or malignant tumours was significantly higher in each of the groups given OXLIN than in the controls, in the groups fed the modified regimen. The groups of females given OXLIN by the normal type of feeding test showed a slight but not significant increase in total tumour incidence. Male rats failed to show an increase in total tumour incidence after the feeding of OXLIN by either regimen.

Table 6 shows the nature of the tumours formed and the dose-response relationships in

Table 4. *Effects of DMBA on tumour incidence and latent periods in female and male rats given a normal or high-fat diet*

Feeding regimen	Group	Effective no.†/group	Mammary gland tumours: Malignant No.‡	Malignant Age§ (wk)	Benign No.‡	Benign Age§ (wk)	Total no.	Tumours of all other organs: Malignant (no.‡)	Tumours of all other organs: Benign (no.‡)	Total no.‖ of rats with tumours
					Females					
Normal	C	50	4 (8)	86	0	—	4 (8)	8 (16)	4 (8)	16 (32)
	DMBA	20	4 (20)	83	2 (10)	64	6 (30)*	1 (5)	4 (20)	10 (50)
Modified	C′	46	3 (7)	95	0	—	3 (7)	9 (19)	7 (15)	15 (33)
	DMBA′	20	6 (30)*	90	1 (5)	83	7 (35)*	9 (45)*	2 (10)	13 (65)
					Males					
Normal	C	43	0	—	1 (2)	90	—	11 (26)	10 (23)	20 (47)
	DMBA	16	0	—	0	—	—	1 (6)	8 (50)	9 (56)
Modified	C′	39	0	—	0	—	—	16 (41)	8 (21)	18 (46)
	DMBA′	17	0	—	0	—	—	6 (35)	3 (18)	8 (47)

†No. of rats surviving to 100 wk plus tumour-bearing animals that died or were killed earlier.
‡Two or more neoplasms in the same tissue in any animal are recorded as one tumour. Values are expressed as percentages in parentheses.
§Mean age at which tumours were detected.
‖Total no. of animals with malignant and/or benign tumours, expressed in parentheses as percentage of the effective no./group.
Values marked with an asterisk differ significantly from the control values: $*P < 0{\cdot}05$.

Table 5. *Total tumour incidence in female and male rats given a normal or high-fat diet*

Feeding regimen	Group	Females			Males		
		Effective no.†/group	With tumours		Effective no.†/group	With tumours	
			No.	(%)		No.	(%)
Normal	C	50	16	32	43	20	47
	OX1	25	12	48	16	8	50
	OX2	20	8	40	18	5	28
	OX3	20	11	55	19	7	37
Modified	C′	46	15	33	39	18	46
	OX1′	19	14	74*	20	9	45
	OX2′	20	12	60*	20	8	40
	OX3′	23	13	57*	21	8	38

†No. of rats surviving to 100 wk plus tumour-bearing animals that died or were killed earlier.
Values marked with an asterisk differ significantly from the control values: *$P < 0.05$.

females. A significant increase in the incidence of cervical sarcomas and mammary adenocarcinomas was seen in some of the experimental groups.

Cervical sarcomas were found in 8 of 127 rats given OXLIN whereas carcinomas were the only malignant cervical tumours observed in some 100 control rats. The incidence of these sarcomas was significantly raised in groups OX1 and OX3, being highest in group OX3. The incidence of malignant plus benign cervical tumours also showed a slight increase with an increase in the dose of OXLIN, being significantly raised in group OX3 but not in groups OX1 or OX2. The cervix, vagina and uterus have been treated as a single organ in this report, because most of the malignant tumours arising in these tissues were anaplastic and had invaded the whole area when detected so that it was difficult to establish their site of origin.

The incidence of malignant mammary tumours was significantly raised in group OX1 but not in groups OX2 or OX3. This is an anomalous finding which merits further investigation.

In all organs other than the mammary gland and cervix there was no significant difference between the percentage of tumours in the experimental and control groups of females. No decrease in the mean latent period of tumour induction was observed in any of the OXLIN groups.

As shown in Table 7, there was an increased incidence of interstitial cell tumours of the testis in males of some OXLIN groups although no increase in the total tumour incidence was observed. The incidence of these testicular tumours showed no apparent dose–response relationship, being significantly raised in groups OX1 and OX2 but not in OX3. No decrease in the latent period of tumour induction was observed.

Figure 1 shows that rats given OXLIN in their diets gained in weight more slowly than their controls during the first year of the experiment. This may have affected the tumour incidence and latent periods in rats of the OXLIN groups.

Administration of OXLIN to mice

Females given the highest dose of OXLIN and the 100-wk modified regimen showed an increased incidence of benign ovarian tumours (Table 8) and of benign tumours in other

Table 6. *Effects of OXLIN on tumour incidence and latent periods in female rats*

Group	Effective no.†/group	Tumours of: Cervix: Malignant: Sarcoma (no.‡)	Cervix: Malignant: Carcinoma (no.‡)	Cervix: Benign (no.‡)	Cervix: Total (no.‡)	Mammary gland: Malignant: No.‡	Mammary gland: Malignant: Age§ (wk)	Mammary gland: Benign: No.‡	Mammary gland: Benign: Age§ (wk)	Mammary gland: Total no.	Other organs: Malignant (no.‡)	Other organs: Benign (no.‡)	Total no.‖ of rats with tumours
C + C′	96	0	2 (2)	3 (3)	5 (5)	7 (7)	90	0	—	7 (7)	16 (16)	8 (8)	31 (32)
All OXLIN groups	127	8 (6)**	3 (2)	2 (2)	13 (10)	17 (13)	99	8 (6)**	101	25 (20)**	29 (23)	15 (12)	71 (56)**
OX1 + OX1′	44	3 (7)*	1 (2)	0	4 (9)	10 (23)**	98	2 (4)	87	12 (27)**	12 (27)	4 (9)	26 (59)**
OX2 + OX2′	40	1 (3)	1 (3)	0	2 (5)	3 (8)	107	3 (7)	102	6 (15)	10 (25)	3 (8)	21 (53)*
OX3 + OX3′	43	4 (9)**	1 (2)	2 (5)	7 (16)*	4 (9)	93	3 (7)	109	7 (16)	7 (16)	8 (19)	24 (56)**

†No. of rats surviving to 100 wk plus tumour-bearing animals that died or were killed earlier.
‡Two or more neoplasms in the same tissue in any animal are recorded as one tumour. Values are expressed as percentages in parentheses.
§Mean age at which tumours were detected.
‖Total no. of animals with malignant and/or benign tumours, expressed in parentheses as a percentage of the effective no./group.
Values marked with asterisks differ significantly from the control values: *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

Table 7. *Effects of OXLIN on tumour incidence and latent periods in male rats*

Group	Effective no.†/group	Tumours of Testis: Malignant: Of Leydig cells (no.‡)	Tumours of Testis: Malignant: Seminomas (no.‡)	Tumours of Testis: Benign (interstitial cell) (no.‡)	Tumours of Testis: Total no.‡ arising in interstitial cells	Other organs: Malignant (no.‡)	Other organs: Benign (no.‡)	Total no.‖ of rats with tumours
C + C′	82	0	1 (1)	1 (1)	1 (1)	27 (33)	18 (22)	38 (46)
All OXLIN groups	114	2 (2)	0	9 (8)*	11 (10)*	32 (28)	13 (11)	45 (39)
OX1 + OX1′	36	2 (6)	0	2 (6)	4 (12)*	12 (33)	5 (13)	17 (47)
OX2 + OX2′	38	0	0	5 (13)*	5 (13)*	10 (26)	3 (8)	13 (34)
OX3 + OX3′	40	0	0	2 (5)	2 (5)	10 (24)	5 (13)	15 (38)

†No. of rats surviving to 100 wk plus tumour-bearing animals that died or were killed earlier.
‡Two or more neoplasms in the same tissue in any animal are recorded as one tumour. Values are expressed as percentages in parentheses.
‖Total no. of animals with malignant and/or benign tumours, expressed in parentheses as a percentage of the effective no./group.
Values marked with an asterisk differ significantly from the control values: $*P < 0{\cdot}05$.

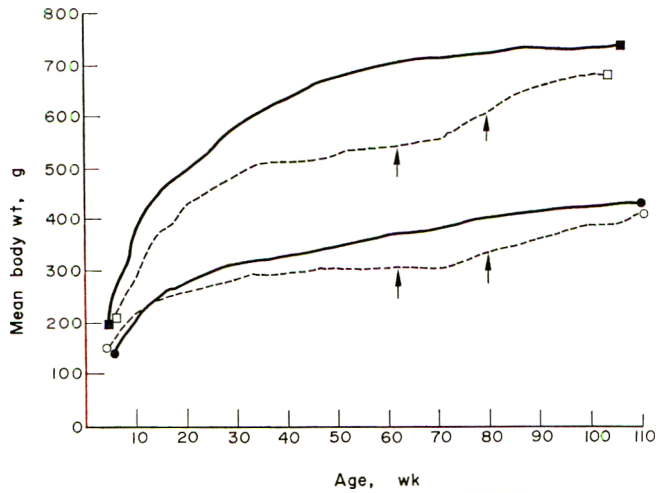


FIG. 1. Curves indicating the mean body-weight recorded throughout the study in female (●; ○) and male (■: □) rats of the control groups given either the normal or modified feeding regimen (●—●; ■—■) and all the OXLIN-fed groups (○- - -○; □- - -□). In the latter groups, OXLIN feeding was discontinued when the animals were 62 wk (for the two lower doses) or 80 wk old, as indicated by arrows.

Table 8. *Effects of OXLIN on tumour incidence in female and male mice*

Feeding regimen	Group	Effective no.†/group	Ovarian tumours		Tumours of all other organs		Total no.§ of mice with tumours
			Malignant (no.‡)	Benign (no.‡)	Malignant (no.‡)	Benign (no.‡)	
			Females				
Normal	C	21	0	3 (14)	0	1 (5)	4 (19)
	OX1	20	0	8 (40)*	1 (5)	0	9 (45)
	OX3	26	0	6 (23)	1 (4)	2 (8)	8 (31)
Modified	C′	49	1 (2)	9 (18)	2 (4)	4 (8)	15 (30)
	OX1′	18	0	4 (23)	0	3 (17)	7 (39)
	OX2′	20	1 (5)	5 (25)	0	4 (20)	7 (35)
	OX3′	20	0	9 (45)*	0	6 (30)*	13 (65)**
			Males				
Normal	C	17	—	—	1 (6)	1 (6)	2 (12)
	OX1	20	—	—	1 (5)	4 (20)	5 (25)
	OX3	21	—	—	2 (10)	1 (5)	3 (15)
Modified	C′	45	—	—	3 (7)	5 (11)	8 (18)
	OX1′	19	—	—	2 (10)	4 (21)	6 (31)
	OX2′	20	—	—	4 (20)	3 (15)	7 (35)
	OX3′	20	—	—	1 (5)	4 (20)	5 (25)

†No. of mice surviving to 80 wk plus tumour-bearing animals that died or were killed earlier.
‡Two or more neoplasms in the same tissue in any animal are recorded as one tumour. Values are expressed as percentages in parentheses.
§Total no. of animals with malignant and/or benign tumours, expressed in parentheses as a percentage of the effective no./group.
Values marked with asterisks differ significantly from control values: *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

organs (lung and cervix). An increased incidence of benign ovarian tumours was also seen in females of group OX1 receiving OXLIN by the normal type of 80-wk feeding test. No increase in malignant tumours was observed in any of the experimental groups of females and the administration of OXLIN had no effect on the tumour incidence of males (Table 8). There was no significant reduction in the age when tumours were detected in either males or females that received OXLIN. In mice the feeding of OXLIN had no effect on the rate of body-weight gain.

DISCUSSION

Many workers have shown that the incidence of tumours induced by a single dose of a carcinogen in the food can be profoundly affected by alterations in the composition of the diet and in the age of the animals when first treated (Clayson, 1962; Gammal *et al.* 1967; Toth, 1968). In the experiments described here, the sensitivity of feeding tests for detecting in female rats the carcinogenic effects of a small single dose of DMBA was increased when the compound was given to rats at an earlier age and a high-fat diet containing 1% polyoxyethylene monostearate was administered. Domsky, Lijinsky, Spencer & Shubik (1963) showed that DMBA was eliminated more slowly from young or newborn animals than from adults. Gammal *et al.* (1967) observed that the administration of a diet containing a high proportion of corn oil to female rats considerably increased both the linoleate content of the mammary tissue and the susceptibility of the rats to mammary carcinogenesis by DMBA, but a diet containing a high proportion of saturated fats did not produce this effect. In the present experiments, the modified diet contained 8% and the normal diet only 1% linoleic acid.

The modified regimen also proved to be more sensitive than the normal method of feeding in demonstrating tumorigenic effects of OXLIN in female rats. The greater sensitivity of the modified regimen was not observed in mice after the feeding of OXLIN; no malignant tumours were produced and the incidence of benign tumours was increased in female mice given OXLIN by either method of feeding.

An increased incidence of malignant tumours in female rats observed after the feeding of OXLIN in the present experiments suggests that oxidation products of linoleate may have carcinogenic properties. It has been known since 1942 that administration of unoxidized polyunsaturated fats promotes tumour induction in the skin and mammary gland of rats and mice (Clayson, 1962; Tannenbaum, 1942), but the tumours produced in the present experiments by the feeding of OXLIN arose at other sites as well as in the mammary gland.

An increased incidence of cervical sarcomas was observed among female rats after the feeding of OXLIN. The incidence of these sarcomas showed a slight increase as the dose of OXLIN became higher. No cervical sarcomas were observed among 100 control rats, and Sharratt, Frazer & Forbes (1964) reported an incidence of only two spontaneous sarcomas of the cervix in 366 rats of this strain. In Wistar rats, these tumours were not observed either by Kim, Clifton & Furth (1960) or by Ratcliffe (1940). The cervical sarcomas observed in these experiments may therefore be induced tumours.

Malignant mammary tumours also increased in incidence but only among females given the lowest dose of OXLIN. This increase in mammary tumours could not be ascribed solely to the promoting effects of unoxidized linoleate, since the amount of unoxidized linoleate in diets containing OXLIN was similar to the amount in the high-fat control diet

used in the modified feeding regimen. (The control diet contained about 8% of linoleate and the diet containing OXLIN about 10% of linoleate.) The unusual dose–response relationship for mammary tumours and the advanced age of the animals when tumours were detected indicates, however, that the effect of OXLIN on mammary tissue is unlike that of a typical carcinogen.

The incidence of benign tumours was increased by OXLIN in organs other than those susceptible to the tumour-promoting effects of unoxidized linoleate. The incidence of interstitial cell tumours of the testis was raised among rats of groups OX1 and OX2, and after administration of OXLIN to female mice, an increased incidence of benign ovarian tumours and of benign tumours in the lung and cervix were observed. In groups treated by the modified regimen, the benign tumours among female mice showed a progressive increase as the dose of OXLIN became higher.

It has been reported by several workers that other products formed during the heating or oxidation of fats produce injection-site sarcomas in the mouse (Swern, Wieder, McDonough, Meranze & Shimkin, 1970; Van Duuren, Langseth, Orris, Teebor, Nelson & Kuschner, 1966; Van Duuren, Melchionne, Blair, Goldschmidt & Katz, 1971; Van Duuren, Nelson, Orris, Palmes & Schmitt, 1963) and an increase of spontaneous tumours after feeding to the rat (Seelkopf & Salfelder, 1962). These compounds include some of the epoxides and hydroxy fatty acids. Local tumours have also been produced by injections of crude lipid extracts, suggesting that other as yet unidentified compounds present may exert carcinogenic activity (Arffmann, 1960; O'Gara, Stewart, Brown & Hueper, 1969). No local tumours were observed in the present experiments after the feeding of OXLIN. There was no increase of tumours in the gastro-intestinal tract which was initially exposed to the lipid peroxides, nor in the liver where most of the metabolism of the hydroperoxide occurs (Freeman, 1964; Glavind & Tryding, 1960). It is therefore probable that breakdown products of OXLIN may be responsible for the observed increase in tumours.

Mitchell & Henick (1962) reported that peroxide values of 0–35 m-moles/kg fat were to be found on analysis of the fats in various foods in western countries and Frazer (1962 & 1968) observed that much higher quantities of peroxides were present in vegetable oils used for cooking in tropical countries such as India, Indonesia and Puerto Rico. Lower peroxide values were found in the saturated fats derived from coconut and palm oils which were generally used in most of Africa and in Singapore. From analysis of the cancer statistics reported by Doll (1969), it would appear that in those tropical areas associated with higher levels of peroxides in dietary fat there is a higher incidence of mammary cancer than in other tropical areas. The annual rates for mammary cancer in females per 100,000 persons is reported to be 41 in Hong Kong, 46 in Bombay and 33 in Puerto Rico, whereas among the Chinese in Singapore and the Africans in Africa the rates are lower, being 19 in Singapore, 10 in Mozambique, 22 in Uganda, 24 in Durban, South Africa, and 25 in Johannesburg, South Africa.

There is also evidence suggesting that a higher incidence of gastric cancer may be associated with increased consumption of heated fats (Dungal, 1958; Higginson, 1968; Seelkopf & Salfelder, 1962).

None of this evidence is conclusive and further studies are required before it can be established that there is a real cancer risk to man from the products of lipid oxidation. The studies reported here do, however, indicate a possible risk, and it may therefore be advisable to add larger quantities of vitamin E or other antioxidants to foodstuffs to reduce the autoxidation of the polyunsaturated fats.

Acknowledgements—This work was supported by the Nuffield Foundation. We are grateful to Dr. M. Sharratt for his helpful advice and criticism, to Dr. I. S. Kiss for microscopic examination of the tumours and to Miss S. Douglas for technical assistance.

REFERENCES

Arcos, J. C., Argus, Mary F. & Wolf, G. (1968). *Chemical Induction of Cancer. Structural Bases and Biological Mechanisms.* Vol. 1. p. 414. Academic Press, New York.

Arffmann, E. (1960). Heated fats and allied compounds as carcinogens: A critical review of experimental results. *J. natn. Cancer Inst.* **25,** 893.

Arffmann, E. (1964). Heated fats and allied compounds as carcinogens. Studied by the newt test. *Acta path. microbiol. scand.* **61,** 161.

Bolland, J. L. (1946). Kinetic studies in the chemistry of rubber and related materials. 1. The thermal oxidation of ethyl linoleate. *Proc. R. Soc.* **A186,** 218.

Clayson, D. B. (1962). *Chemical Carcinogenesis.* J. & A. Churchill Ltd., London.

Dickey, F. H., Cleland, G. H. & Lotz, C. (1949). Organic peroxides in the induction of mutations. *Proc. natn. Acad. Sci. U.S.A.* **35,** 581.

Doll, R. (1969). The geographical distribution of cancer. *Br. J. Cancer* **23,** 1.

Domsky, I. I., Lijinsky, W., Spencer, Kay & Shubik, P. (1963). Rate of metabolism of 9,10-dimethyl-1,2-benzanthracene in newborn and adult mice. *Proc. Soc. exp. Biol. Med.* **113,** 110.

Dulog, L. & Burg, K. H. (1963). The autoxidation of the methyl ester of linoleic acid. *Dt. Farben-Z.* **17,** 21.

Dungal, N. (1958). Cancer in Iceland. In *Cancer.* Vol. 3. Edited by R. W. Raven. p. 262. Butterworth & Co. Ltd., London.

Farmer, E. H. & Sutton, D. A. (1943). The course of autoxidation reactions in polyisoprenes and allied compounds. Part IV. The isolation and constitution of photochemically-formed methyl oleate peroxide. *J. chem. Soc.* Pt. 1, 119.

Fisher, R. A. (1954). *Statistical Methods for Research Workers.* p. 96. Oliver & Boyd, Edinburgh.

Fisher, W. D. & Wilbur, K. M. (1954). Effect of irradiated fatty acids on the viscosity of nucleic acids in the rat. *J. Elisha Mitchell scient. Soc.* **70,** 124.

Fränz, J. & Cole, B. T. (1962). Effects of ultraviolet-irradiated methyl linolenate on cell division and respiration in *Saccharomyces cerevisiae. Archs Biochem. Biophys.* **96,** 382.

Frazer, A. C. (1962). The possible role of dietary factors in the aetiology and pathogenesis of sprue, coeliac disease and idiopathic steatorrhoea. *Proc. Nutr. Soc.* **21,** 42.

Frazer, A. C. (1968). *Malabsorption Syndromes.* p. 89. Heinemann Ltd., London.

Freeman, I. P. (1964). The metabolism of linoleic acid hydroperoxide. *Chem. Ind.* **28,** 1543.

Gammal, E. B., Carroll, K. K. & Plunkett, E. R. (1967). Effects of dietary fat on mammary carcinogenesis by 7,12-dimethylbenz-(a)-anthracene in rats. *Cancer Res.* **27,** 1737.

Glavind, J. & Arffmann, E. (1970). The possible carcinogenic properties of altered lipids. A study of purified compounds by the newt test. *Acta path. microbiol. scand. Sect. A* **78,** 345.

Glavind, J. & Tryding, N. (1960). On the digestion and absorption of lipoperoxides. *Acta physiol. scand.* **49,** 97.

Gruenstein, M., Meranze, D. R., Thatcher, D. & Shimkin, M. B. (1966). Carcinogenic effects of intragastric 3-methylcholanthrene and 7,12-dimethylbenze[*a*]anthracene in Wistar and Sprague-Dawley rats. *J. natn. Cancer Inst.* **36,** 483.

Higginson, J. (1968). Distribution of different patterns of cancer. *Israel J. med. Sci.* **4,** 457.

Holman, R. T. & Greenberg, S. I. (1958). The toxicities of methyl oleate peroxide. *J. Am. Oil Chem. Soc.* **35,** 707.

Horgan, V. J., Philpot, J. S. L., Porter, B. W. & Roodyn, D. B. (1957). Toxicity of autoxidized squalene and linoleic acid, and of simpler peroxides in relation to toxicity of radiation. *Biochem. J.* **67,** 551.

Huggins, C., Grand, Lorraine C. & Brillantes, Filomena P. (1961). Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. *Nature, Lond.* **189,** 204.

Huggins, C., Morii, S. & Grand, L. C. (1961). Mammary cancer induced by a single dose of polynuclear hydrocarbons: Routes of administration. *Ann. Surg.* **154,** Suppl. 6, p. 315.

Kaunitz, H. (1953). Studien über die Ernährung von Ratten mit hochoxydierten Fetten. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **220,** 16.

Kim, U., Clifton, K. H. & Furth, J. (1960). A highly inbred line of Wistar rats yielding spontaneous mammosomatotropic pituitary and other tumours. *J. natn. Cancer Inst.* **24,** 1031.

Mitchell, J. H., Jr. & Henick, A. S. (1962). Rancidity in food products. In *Autoxidation and Antioxidants.* Vol. II. Edited by W. O. Lundberg. p. 543. Interscience Publishers, New York.

O'Gara, R. W., Stewart, L., Brown, J. & Hueper, W. C. (1969). Carcinogenicity of heated fats and fat fractions. *J. natn. Cancer Inst.* **42,** 275.

Ralston, A. W. (1948). *Fatty Acids and Their Derivatives*. p. 127. John Wiley & Sons, London.
Rao, B. Y. (1960). Nutritional aspects of heated, oxidized and polymerized fats and oils. *J. scient. ind. Res.* **A19,** 430.
Ratcliffe, H. L. (1940). Spontaneous tumours in two colonies of rats of the Wistar Institute of Anatomy and Biology. *Am. J. Path.* **16,** 237.
Ross, M. H. (1959). Protein, calories and life expectancy. *Fedn Proc. Fedn Am. Socs exp. Biol.* **18,** 1190.
Seelkopf, C. & Salfelder, K. (1962). Tierversuche zur Frage cancerogener Eigenschaften einiger Epoxyde in überhitzten Fetten. *Z. Krebsforsch.* **64,** 459.
Sharratt, M., Frazer, A. C. & Forbes, O. C. (1964). Study of the biological effects of benzoyl peroxide. *Fd Cosmet. Toxicol.* **2,** 527.
Stillwell, E. F., Maroney, S. P. & Wilbur, K. M. (1959). Effect of irradiated fatty acids on the growth of *Escherichia coli*. *J. Bact.* **77,** 510.
Swern, D., Wieder, R., McDonough, M., Meranze, D. R. & Shimkin, M. B. (1970). Investigation of fatty acids and derivatives for carcinogenic activity. *Cancer Res.* **30,** 1037.
Tame, M. J. & Dils, R. (1967). Fatty acid synthesis in intestinal mucosa of guinea-pig. *Biochem. J.* **105,** 709.
Tannenbaum, A. (1942). The genesis and growth of tumours. III. Effects of a high fat diet. *Cancer Res.* **2,** 468.
Toth, B. (1968). A critical review of experiments in chemical carcinogenesis using newborn animals. *Cancer Res.* **28,** 727.
Van Arkel, G. A. (1958). Modification of Ultraviolet and Formaldehyde Mutagenesis in Aspergillus nidulans. Ph.D. Thesis, Utrecht.
Van Duuren, B. L., Langseth, L., Orris, L., Teebor, G., Nelson, N. & Kuschner, M. (1966). Carcinogenicity of epoxides, lactones, and peroxy compounds. IV. Tumor response in epithelial and connective tissue in mice and rats. *J. natn. Cancer Inst.* **37,** 825.
Van Duuren, B. L., Melchionne, S., Blair, R., Goldschmidt, B. M. & Katz, C. (1971). Carcinogenicity of isosters of epoxides and lactones: Aziridine ethanol, propane sultone, and related compounds. *J. natn. Cancer Inst.* **46,** 143.
Van Duuren, B. L., Nelson, N., Orris, L., Palmes, E. D. & Schmitt, F. L. (1963). Carcinogenicity of epoxides, lactones, and peroxy compounds. *J. natn. Cancer Inst.* **31,** 41.
Wilbur, K. M., Wolfson, Nancy, Kenaston, Carolyn B., Ottolenghi, A., Gaulden, Mary E. & Bernheim, F. (1957). Inhibition of cell division by ultraviolet irradiated unsaturated fatty acid. *Expl Cell Res.* **13,** 503.

La sensibilité des tests d'alimentation dans la détection des propriétés carcinogènes des produits chimiques: Etude du 7,12-diméthylbenz[*a*]anthracène et du linoléate oxydé

Résumé—La sensibilité des tests d'alimentation utilisés pour détecter les propriétés carcinogènes d'une petite dose de 7,12-diméthylbenz[*a*]anthracène chez le rat femelle a augmenté quand ce produit était administré à de jeunes animaux et associé à un régime riche en matière grasse contenant 1% de monostéarate de polyoxyéthylène. Ces modifications du régime alimentaire ont également permis de détecter plus facilement les propriétés oncogènes du linoléate oxydé chez le rat femelle.

Une plus grande proportion de rats femelles ont présenté des sarcomes du col de l'utérus et des adénocarcinomes mammaires et la fréquence des tumeurs de la cellule interstitielle du testicule a augmenté chez les rats mâles après que l'on eût ajouté du linoléate oxydé au régime de ces animaux. La consommation de linoléate oxydé a provoqué chez les souris femelles une augmentation des tumeurs bénignes, ovariennes et autres.

L'article examine le risque de danger que l'auto-oxydation des lipides peut présenter pour la population.

Empfindlichkeit von Fütterungsversuchen für die Feststellung carcinogener Eigenschaften von Chemikalien: Untersuchung von 7,12-Dimethylbenz[*a*]anthrazen und oxydiertem Linoleat

Zusammenfassung—Die Empfindlichkeit von Verfütterungsversuchen für die Feststellung der carcinogenen Eigenschaften einer kleinen Dosis von 7,12-Dimethylbenz[*a*]anthrazen bei weiblichen Ratten wurde erhöht, wenn die Verbindung den Ratten in jüngerem Lebensalter verabreicht und wenn ein Futter hohen Fettgehalts mit 1% Polyoxyäthylenmonostearat verwendet wurde. Solche Modifikationen der Fütterungsweise erlaubten auch, die tumorerzeugenden Eigenschaften von oxydiertem Linoleat bei weiblichen Ratten leichter festzustellen.

Eine grössere Anzahl weiblicher Ratten wies cervikale Sarkome und mammäre Adenocarcinome nach dem Zusatz von oxydiertem Linoleat zu ihrem Futter auf, und bei männlichen Ratten war die Häufigkeit von Interstitialzelltumoren der Testes erhöht. Die Verfütterung von oxydiertem Linoleat an Mäuse führte zu einer vermehrten Häufigkeit benigner Ovarialtumoren und anderer benigner Tumoren bei den weiblichen Tieren. Die mögliche Gefährdung der Bevölkerung durch die Autoxydation von Lipiden wird besprochen.

Fd Cosmet. Toxicol. Vol. 11, pp. 459–462. Pergamon Press 1973. Printed in Great Britain

SHORT PAPERS

Reproduction Study with Formaldehyde and Hexamethylenetetramine in Beagle Dogs

H. HURNI and H. OHDER

Laboratory of Bio-medical Research, Tierfarm Sisseln, Switzerland

(*Received 22 November 1972*)

Summary—The effects of hexamethylenetetramine (HMT) and formaldehyde (FA) on reproduction were studied in 51 beagle bitches (mean body weight 12 kg). The dogs were fed on days 4–56 after mating with a daily ration of 300 g dry pellets containing either FA or HMT at levels of 125 or 375 ppm FA or 600 or 1250 ppm HMT. Control animals were given untreated pellets. The treatments did not affect the pregnancy rate, the weight gain of the pregnant beagles, the length of gestation or the size of the 44 litters. The percentage of still-born pups showed a slight increase and the weight gain of the pups and the survival to weaning were slightly impaired by the higher dietary level of HMT. No malformations (either internal or skeletal) were observed in any of the 264 live-born and 20 still-born pups.

Introduction

The use of hexamethylenetetramine (HMT) as an antimicrobial agent in food has caused concern, mainly because of a lack of toxicological data and a suspected carcinogenic action (Joint FAO/WHO Expert Committee on Food Additives, 1962). Subsequent short- and long-term studies revealed a very low toxicity and no evidence of carcinogenicity in mice and rats (Brendel, 1964; Della Porta, Colnaghi & Parmiani, 1968 & 1970; Natvig, Andersen & Wulff Rasmussen, 1971). Two reproduction studies in the rat showed no effect on fertility or the incidence of abnormalities in the litters (Della Porta, Cabral & Parmiani, 1970; Natvig *et al.* 1971), but no reproduction studies in dogs as requested by the Joint FAO/WHO Expert Committee on Food Additives (1967) have been published.

The present study was undertaken to investigate the effects of the oral administration of HMT to beagle dogs during gestation. As the toxicological effects of HMT appear to be due to the liberation of formaldehyde (FA), other groups of animals were treated with FA.

Experimental

Animals. The 51 bitches used were from our closed breeding colony of beagle dogs and had already successfully raised one or more litters. When 9 wk old, all animals had had a combined vaccination against distemper, hepatitis and leptospiroses, and a booster vaccination was given yearly. A dose of piperazine citrate was administered at 4 and 6 wk of age as well as 4 and 6 wk after each mating. Breeding bitches were housed in groups of four or five in indoor cubicles connected to an outdoor run, each pregnant bitch being transferred to an air-conditioned whelping room 1 wk before term.

Diet. The dogs were fed exclusively on dog pellets (Nafag AG, Gossau SG, Switzerland) and were fasted each Saturday. The daily ration for bitches was 300 g, which was reduced

to 200 g 1 wk before term. No food was offered on the day of parturition. The animals were given 300 g on the following day, and 300 g twice daily for the next 3 wk. From wk 4 to the end of wk 12, the mother was allowed 250 g twice daily, while the pups in temporary isolation received two feeds of 150 g daily. Pups of inadequately lactating mothers were supported with reconstituted cows' milk.

Experimental design and conduct. Commercial grade HMT and FA were used, FA being provided as a 40% solution. HMT was given at dietary levels of 600 and 1250 ppm and FA at dietary levels of 125 and 375 ppm. Solutions containing the required daily dosage in 2·0 ml were prepared weekly and sprayed on the pellets prior to feeding. The pellets were promptly consumed (within 5–10 min) before the FA had a chance to volatilize. The treatment was initiated 4 days after mating and lasted until day 56, at which time the dog was transferred to the whelping room. The dosage regimes and group sizes are detailed in Table 1. The bitches were weighed at weekly intervals throughout pregnancy and lactation. The pups were weighed at birth and twice weekly thereafter. They were inspected for visible defects immediately after birth and after 8 wk. Stillborn pups and those lost before weaning were autopsied and examined for internal and skeletal abnormalities.

Table 1. *Dosage schedules and fertility of female beagles fed diets containing FA or HMT during gestation*

	Treatment					
Diet	Dietary level (ppm)	Dose (mg/kg body weight/day)	No. of mated bitches	No. of pregnant bitches	No. of litters	Mean length of gestation (days)
Control	0·0	0·0	11	9	9	65·8
FA	125	3·1	11	10	10	63·6
	375	9·4	10	9	9	64·7
HMT	600	15	9	8	8	63·3
	1250	31	10	9	8	63·5

Further observation. Of the pups weaned in this experiment, it was possible to observe 212 for longer periods. Two dogs were observed for 5 months, 36 for 6, 81 for 7, 64 for 8 and 11 for 9 months, while the remaining 18 dogs were used for breeding purposes and are still in our beagle colony.

Results and Discussion

The treatments did not affect the pregnancy rate (Table 1). One pregnant bitch in the group given the higher level of HMT was severely injured in a fight and had to be eliminated. The body weight increased regularly during pregnancy in all groups and the duration of gestation was unaffected by the treatments (Table 1).

Table 2 presents the mean litter sizes in the various test and control groups, as well as the mean numbers of pups of each sex born live and weaned. The mean litter size was within the normal range for all groups, demonstrating that fecundity was not affected by treatment. In the group that received the higher dose of HMT, the percentage of stillborn pups was higher than in any of the other groups, mainly because in one litter of nine pups only two were born alive. No skeletal or any other malformations were observed in any of the stillborn pups.

Table 2. *Fecundity of female beagles given FA or HMT orally during gestation*

Diet (ppm)	No. of litters	Total pups at birth	Total live pups at birth	Total pups at weaning	Mean pups/litter					
					Total births		Live births		At weaning	
					Male	Female	Male	Female	Male	Female
Control	9	60	56	49	3·1	3·6	2·9	3·3	2·3	3·1
FA (125)	10	54	50	50	2·6	2·8	2·4	2·6	2·4	2·6
FA (375)	9	64	64	60	4·2	2·9	4·2	2·9	3·9	2·8
HMT (600)	8	50	48	45	3·3	3·0	3·3	2·8	3·1	2·5
HMT (1250)	8	56	46	33	3·7	3·3	2·6	3·1	1·5	2·6

Data on the body weight of the pups is summarized in Table 3. During the first month there was a retardation of growth in the group given the higher dose of HMT, coinciding with an increase in mortality. In the same group the percentage of pups that survived to weaning was lower than in the other groups.

Table 3. *Mean body weight of pups born to beagles given FA or HMT orally during gestation*

	Mean body weight (g) of pups* at wk								
Diet (ppm)	0†	1	2	3	4	5	6	7	8
Control	321	547	818	1078	1264	1601	2020	2449	2957
FA (125)	308	526	755	987	1247	1512	1816	2263	2712
FA (375)	294	467	706	944	1166	1429	1741	2145	2587
HMT (600)	290	508	753	1046	1295	1499	1805	2324	2780
HMT (1250)	294	421	648	889	1125	1425	1742	2160	2698

*In each group values are means for all the pups surviving at the time stated.
†Weight at birth.

All the dogs observed for a more prolonged period have been normal in behaviour, appearance, motility and muscular co-ordination. The dogs observed up to 9 months were used for various other investigations, for which they were eventually killed and autopsied. No malformations were found. The 18 dogs transferred to the breeding colony have been under observation for nearly 2 yr. Neither these adults nor their litters have shown any signs of physiological or skeletal abnormalities or disorders of reproduction.

The present study thus revealed no teratogenic action of FA or HMT. Even with the higher dose of HMT given, corresponding to a daily intake of 0·031 g/kg body weight, the adverse effect on reproduction was slight.

REFERENCES

Brendel, R. (1964). Untersuchungen an Ratten zur Verträglichkeit von Hexamethylenetetramin. *Arzneimittel-Forsch.* **14,** 51.

Della Porta, G., Cabral, J. R. & Parmiani, G. (1970). Studio della tossicità transplacentare e di cancerogenesi in ratti trattati con esametilentetramina. *Tumori* **56,** 325.

Della Porta, G., Colnaghi, Maria I. & Parmiani, G. (1968). Non-carcinogenicity of hexamethylenetetramine in mice and rats. *Fd Cosmet. Toxicol.* **6,** 707.

Joint FAO/WHO Expert Committee on Food Additives—Sixth Report (1962). Evaluation of the Toxicity of a Number of Antimicrobials and Antioxidants. *Tech. Rep. Ser. Wld Hlth Org.* **228.**

Joint FAO/WHO Expert Committee on Food Additives—Tenth Report (1967). Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation: Some Emulsifiers and Stabilizers and Certain Other Substances. *Tech. Rep. Ser. Wld Hlth Org.* **373.**

Natvig, H., Andersen, J. & Wulff Rasmussen, E. (1971). A contribution to the toxicological evaluation of hexamethylenetetramine. *Fd Cosmet. Toxicol.* **9,** 491.

Fd Cosmet. Toxicol. Vol. 11, pp. 463–466. Pergamon Press 1973. Printed in Great Britain

Metabolism of Aflatoxin B_1 by the Guinea-Pig

MARY T. KOES, L. J. FORRESTER and H. D. BROWN
Cancer Research Center, Columbia, Missouri 65201, USA

(*Received 28 August 1972*)

Summary—Aflatoxin B_1 was given by ip injection to year-old female guinea-pigs and 6 hr later aflatoxin B_1 and its metabolites were recovered and isolated chromatographically. Approximately 14% of the administered toxin was represented by the isolated compounds. Aflatoxin B_1 and M_1 each accounted for approximately 3%, and the remaining 94% was a product that migrated with the solvent front in a chloroform–acetone thin-layer chromatograph. This major metabolite exhibited a blue-green fluorescence and absorbed at 270 nm in methanolic solution. The compound appeared stable, since it rechromatographed as a single entitiy.

Introduction

Patterson & Allcroft (1970) and Patterson, Roberts & Allcroft (1969) reported that the livers of the chick, duckling, guinea-pig and mouse metabolized aflatoxin B_1 almost completely under *in vitro* incubation conditions. This transformation does not occur in the calf, goat, pig, rat and sheep. Routes of aflatoxin metabolism are thought to be hydroxylation and *O*-demethylation (Wogan, 1969). Such reactions are commonly associated with the hepatic mixed-function oxidation system, a primary site of 'detoxication'.

Patterson *et al.* (1969) noted an association between the basal aniline-hydroxylase levels and the *in vitro* rate of B_1 metabolism, but there was no trend in the more resistant species toward higher basal levels of microsomal hydroxylase or greater rates of aflatoxin B_1 metabolism.

Our studies were conducted with year-old female guinea-pigs as a pilot system. The guinea-pig is particularly susceptible to the toxic effects of aflatoxin and also provides a highly active mixed-function oxidation system under control conditions. It is thus an appropriate model to use for observing the hypothesized interaction between mixed-function oxidase and aflatoxin B_1. The effect of *in vivo* treatment with aflatoxin B_1 on NADPH oxidase, ferricyanide reductase, cytochrome *P*-450 and cytochrome b_5 activities in control and experimental guinea-pig microsomal fractions was determined.

Experimental

Aflatoxin metabolism. Year-old female guinea-pigs were injected ip with 5 mg aflatoxin B_1/kg body weight, dissolved in dimethylformamide (DMF). Animals were killed 6 hr after injection and the liver was divided for the study of aflatoxin metabolism and the assay of mixed-function oxidation activity. Aflatoxins were extracted from liver with acetone–chloroform–water, 38:58:4, by vol. (Purchase & Steyn, 1969). The extracted aflatoxin residue was evaporated to dryness in a vacuum rotary evaporator and then redissolved in

0·5 ml benzene–acetonitrile, 98:2, v/v, for application to silica-gel thin-layer chromatography (TLC) plates (Brinkman Instruments, Inc., Westbury, N.Y.). Plates were developed in chloroform–acetone, 90:10, v/v, and metabolites were identified under long wave ultraviolet light by comparison with reference standards supplied by the Southern and Northern Regional USDA Laboratories. Aflatoxins were recovered from silica gel by scraping and elution with methanol prior to quantitation in an Aminco–Bowman spectrophotofluorometer. The activation and fluorescent wavelengths of aflatoxin B_1 were 370 and 430 nm, while those of M_1 were 370 and 440 nm, respectively. An approximate quantitative estimation of compound "X" was obtained at the same activation and fluorescent wavelengths as B_1. The B_1 standard curve was linear from 10 to 60 ng/ml and served also as a reference for the other metabolites.

Cell fractionation. The remainder of the liver tissue was homogenized in 3 vols 1·15% KCl in a Waring Blender for 2 min and then further homogenized for 3 min in a size C Thomas Tube using a motor-driven pestle. This suspension was centrifuged for 25 min at 9000 **g**. The supernatant was retained for further centrifugation at 105,000 **g** for 2 hr to isolate the microsomal fraction. Microsomal pellets were assayed for activities of cytochrome *P*-450, cytochrome b_5, NADPH oxidase and ferricyanide reductase.

Enzyme assays. All enzyme activities were measured spectrophotometrically using a Cary 15 spectrophotometer.

NADPH-oxidase activity was determined in a reaction mixture containing 0·1 ml of the liver microsome fraction, 0·2 M-potassium phosphate buffer (pH 7·4), 100 μmoles nicotinamide and 0·25 mole NADPH in a total volume of 2·0 ml (Gillette, Brodie & La Du, 1957). The volume of the reaction mixture was adjusted by the addition of the potassium phosphate buffer and NADPH was omitted from the blank determination. The rate of decrease in adsorption at 340 nm was used as a measure of NADPH oxidase.

Microsomal ferricyanide reductase was measured in a reaction mixture containing 0·1 ml of the liver microsomal fraction, 200 nmoles potassium ferricyanide and 300 nmoles NADPH in a total volume of 3·0 ml (Williams & Kamin, 1962). A potassium phosphate buffer (0·05 M; pH 7·7) was used to provide a final volume of 3 ml, ferricyanide was not added to the blank, and NADPH was added last to initiate the reaction. Decrease in absorbancy at 420 nm was used to determine activity.

Microsomal *P*-450 was assayed by measuring the difference spectra of the preparation following the procedure of Omura & Sato (1964) and Sato (1966). Cytochrome b_5 was measured in a 3·0 ml reaction containing 800 μg protein (105,000 **g** fraction) and 2% NADH (J. R. Fouts, personal communication 1967).

The Biuret procedure was used to determine protein content of the microsomal fraction.

Results and Discussion

Metabolism of aflatoxin B_1. About 14% of the original dose was recovered as B_1 or its metabolites 6 hr after the administration of aflatoxin B_1. The quantitative distribution of these compounds is shown in Table 1. The value obtained for compound "X" must be regarded as an approximate estimate. The amount of M_1, the hydroxylated derivative of B_1, was similar to the quantity of B_1. The other metabolite, "X", which migrated with the solvent front in a chloroform–acetone, 90:10, v/v, system constituted the major product and demonstrated a blue-green fluorescence and an absorption peak at 270 nm in methanolic solution. The possibility that this compound was contaminated with impurities migrating at the solvent front was considered, and the compound was recovered for rechromato-

Table 1. In vivo *metabolism of aflatoxin B_1 in guinea-pig liver*

Metabolite	Concentration in liver	
	μg/g liver	% of total metabolites
B_1	0·681	3·14
M_1	0·678	3·13
X	20·300	93·72
Total	21·660	

Values represent the means of duplicate determinations on two animals.

graphy in a more polar (methanol) system. Rechromatography resulted in the appearance of one spot with an R_F of 0·67 and the characteristic blue-green fluorescence. TLC, isolation, recovery and rechromatography of natural products of aflatoxin metabolism usually result in the oxidative degradation of that metabolite to several components (L. S. Lee, personal communication 1971). However, compound "X" is apparently quite stable as evidenced by its rechromatography as a single entity.

Patterson & Roberts (1970) have also reported that the *in vitro* metabolism of aflatoxin B_1 results in the production of an unidentified but nonfluorescent compound, which they have tentatively designated as a hemiacetal on the basis of its absorption at 400 nm in a 50% methanolic solution. It is interesting to note that aflatoxin B_1 is metabolized to two apparently different compounds in the *in vitro* and *in vivo* states. Patterson & Roberts (1970) have associated the loss in hepatotoxicity during incubation with the transformation of B_1 to the hemiacetal, which is known to have a very low level of acute toxicity. Compound "X" could be an epoxide and have substantially greater toxicity than the hemiacetal. However, the TLC migration characteristics of "X" would preclude such an epoxide structure. Since "X" migrates with the solvent front, it is thought to be less polar than either M_1 or B_1 and may represent the demethoxylated derivative of B_1. Additional investigation is necessary to determine the chemical nature of this abundant product as well as its toxicity.

Table 2. *Effect of aflatoxin B_1 metabolism on some mixed-function oxidase activities in guinea-pig liver*

Animal no.	Aflatoxin B_1 treatment	Activity (ΔOD/g protein)				
		Cytochrome		NADPH oxidase	Ferricyanide reductase	Cytochrome b_5
		P-450	*P*-420			
1	—	4·0	10·0	2·5	12·0	6·6
2	—	4·9	18·0	2·9	14·0	7·0
3	+	5·8	18·0	2·6	16·0	9·0
4	+	4·8	14·0	3·1	15·0	7·0

Values represent means of triplicate determinations.

Mixed-function oxidase activities. Under the conditions of our *in vivo* experiments, activities of certain mixed-function oxidase components were virtually unaffected by aflatoxin B_1 treatment (Table 2). It is possible that continuous feeding of B_1 would promote changes in activity undetectable after short-term dosage. Furthermore, evaluation of the actual effect of aflatoxin might be determined more rigorously in an *in vitro* system which is actively metabolizing B_1.

Acknowledgement—This investigation was supported by Public Health Service Research Grant No. CA-08023 and General Research Support Grant No. FR-05618 from the National Institutes of Health.

REFERENCES

Gillette, J. R., Brodie, B. B. & La Du, B. N. (1957). The oxidation of drugs by liver microsomes: On the role of TPNH and oxygen. *J. Pharmac. exp. Ther.* **119,** 532.

Omura, T. & Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. biol. Chem.* **239,** 2370.

Patterson, D. S. P. & Allcroft, Ruth (1970). Metabolism of aflatoxin in susceptible and resistant animal species. *Fd Cosmet. Toxicol.* **8,** 43.

Patterson, D. S. P. & Roberts, B. A. (1970). The formation of aflatoxins B_{2a} and G_{2a} and their degradation products during the *in vitro* detoxification of aflatoxin by livers of certain avian and mammalian species. *Fd Cosmet. Toxicol.* **8,** 527.

Patterson, D. S. P., Roberts, B. A. & Allcroft, Ruth (1969). Aflatoxin metabolism. *Fd Cosmet. Toxicol.* **7,** 277.

Purchase, I. F. H. & Steyn, M. (1969). The metabolism of aflatoxin B_1 in rats. *Br. J. Cancer* **23,** 800.

Sato, R. (1966). State of existence and function of P-450 in microsomal drug hydroxylation. In *Biological and Chemical Aspects of Oxygenases.* Edited by K. Bloch and O. Hayaishi. p. 195. Maruzen Company, Tokyo.

Williams, C. H., Jr. & Kamin, H. (1962). Microsomal triphosphopyridine nucleotide–cytochrome *c* reductase of liver. *J. biol. Chem.* **237,** 587.

Wogan, G. N. (1969). Metabolism and biochemical effects of aflatoxins. In *Aflatoxin.* Edited by L. A. Goldblatt. p. 151. Academic Press, New York.

Fd Cosmet. Toxicol. Vol. 11, pp. 467–470. Pergamon Press 1973. Printed in Great Britain

Susceptibility of Vitamin A-deficient Rats to Aflatoxin

G. SRIRANGA REDDY, T. B. G. TILAK and D. KRISHNAMURTHI

National Institute of Nutrition, Indian Council of Medical Research, Hyderabad 500007, A.P., India

(*Received 28 November 1972*)

Summary—Weanling albino rats were divided into two groups each of six males and six females. They were maintained on a vitamin A-free diet, one group being fed *ad lib.* and the second being pair-fed with the first. Rats in this second group were given an oral supplement of 100 IU vitamin A/animal/day. At the end of a feeding period of 9 wk, a crystalline preparation of aflatoxin was administered in a single ip dose of 3·5 mg/kg body weight. Male rats in the first group showed an increased susceptibility to the toxin as evidenced by rapid mortality and severe liver damage, midzonal in distribution. Their hepatic stores of vitamin A were found to have been severely depleted. Vitamin A-supplemented male rats showed only mild hepatic damage of the periportal parenchyma. Liver damage was minimal in female rats of both groups and was unaffected by the amount of vitamin A stored in the liver.

Introduction

The toxic effects of aflatoxins in laboratory animals and birds have been shown to be affected by dietary protein (Madhavan & Gopalan, 1965; Madhavan, Rao & Tulpule, 1967), fat (Hamilton, Hsi-Tang Tung, Harris, Gainer & Donaldson, 1972; Smith, Hill & Hamilton, 1971) and lipotrophic factors (Rogers & Newberne, 1971). Compared with aflatoxin-treated rats on a normal diet, a decreased incidence of hepatomas and an increase in the occurrence of colonic carcinomas were reported in vitamin A-deficient rats given aflatoxin (Newberne & Rogers, 1972). It was reported earlier that aflatoxicosis in animals (Allcroft & Lewis, 1963; Keyl, Booth, Masri, Gumbmann & Gagne, 1970) and in birds (Carnaghan, Lewis, Patterson & Allcroft, 1966) reduced hepatic and serum levels of vitamin A. The present communication deals with the response of vitamin A-deficient rats to a single toxic dose of aflatoxin.

Experimental

Animals and diet. Weanling albino rats of both sexes weighing between 28 and 50 g were selected from a limited number of litters from the stock colony of the National Institute of Nutrition. They were divided into two groups matched in respect of litter mates and body weights and consisting in each case of six males and six females. The rats of both the groups were maintained on a vitamin A-free diet consisting of vitamin A-free casein (20%), maize starch (60%), cottonseed oil (5%), vitamin mixture (1%), salt mixture (4%) and sucrose (10%). The casein was refluxed with ethanol for 6 hr, extracted with ether and oven-dried at 110°C for 48 hr. Each gram of vitamin mixture contained vitamin K (0·5 mg), thiamine hydrochloride (0·5 mg), riboflavin (1·0 mg), pyridoxine hydrochloride (0·4 mg), pantothenic acid (4·0 mg), niacin (4·0 mg), inositol (25·0 mg), *p*-aminobenzoic acid (10·0 mg), vitamin B_{12} (2·0 μg), biotin (0·02 mg), folic acid (0·2 mg) and vitamin E (10 IU). In addition choline chloride (100 mg/100 g diet) and vitamin D (12·5 μg/100 g diet) were added.

Animals in group 1 received the diet *ad lib.* and each animal of this group had its pair-fed control rat of the corresponding sex in group 2. The latter group of animals received in addition an oral supplement of vitamin A at the rate of 100 IU/rat/day throughout the experiment. The rats were housed individually and were given water *ad lib.*

Treatment. Each rat was weighed weekly. After 9 wk on the test diets, each animal was given ip a mixture of crystalline aflatoxins dissolved in propylene glycol in a concentration of 1 mg/ml. The crystalline aflatoxin preparation, given in a dose of 3·5 mg/kg body weight, included aflatoxins B_1 (44%), G_1 (44%) and B_2 and G_2 (2%). Some 40 hr after the toxin was administered, all the male rats in group 1 died. Although no adverse effects were noted in any other animals, the experiment was terminated at this point, the rest of the animals in both groups being killed. The liver from each of the animals that died or was killed was removed and weighed immediately.

Determination of liver levels of vitamin A. Part of the liver from each animal was immediately frozen and preserved for not more than 1 wk at −20°C for the estimation of vitamin A by the Carr-Price method (Embree, Ames, Lehman & Harris, 1957).

Histology. The rest of each liver was fixed in 10% neutral buffered formol-saline. Paraffin blocks of the tissue were sectioned at 5 μ and stained with haematoxylin and eosin, with periodic acid–Schiff (PAS) reagent before and after salivary digestion, and with Masson's trichrome stain.

Results

Rats in both groups continued to gain weight until the toxin was administered at the end of 9 wk. The body weights of the male and female rats of group 1 were comparable with those of the corresponding pair-fed controls of group 2 (Table 1). Hepatic stores of vitamin A in the rats of group 1 were significantly lower ($P < 0{\cdot}001$) than those of the animals of group 2, but within each group, the hepatic vitamin A levels were comparable in males and females (Table 1).

As already mentioned, vitamin A-deficient male rats (group 1) died about 40 hr after aflatoxin administration. The vitamin A-deficient females (group 1) and all the vitamin A-supplemented animals (group 2) showed no adverse reactions.

Table 1. *Mean body weights and hepatic vitamin A levels in rats fed diets deficient in or supplemented with vitamin A for 9 wk*

Group	Dietary feeding regime	Vitamin A intake (IU/rat/day)	Mean body weight (g)		Vitamin A (IU/whole liver)
			Initial	Terminal	
			Males		
1	*Ad lib.*	0	45·5 ± 1·94	226·7 ± 8·26	36·5 ± 6·16*†
2	Pair-fed	100	45·7 ± 2·42	201·0 ± 10·77	2128·1 ± 153·56
			Females		
1	*Ad lib.*	0	40·0 ± 2·34	136·8 ± 2·40	18·6 ± 3·42*
2	Pair-fed	100	37·5 ± 2·14	130·0 ± 4·11	2299·1 ± 111·49

†Mean values for five rats, since in one rat, liver vitamin A could not be estimated.
Values are means ± SEM for determinations in groups of six animals except where indicated otherwise and those marked with an asterisk differ significantly (paired *t* test) from those for the corresponding vitamin A-supplemented, pair-fed group: *$P < 0{\cdot}001$.

Liver histology

The livers of the vitamin A-deficient male rats (group 1) were grossly congested, but those of the females in this group and both sexes of the other group appeared normal. Histologically, the livers of the male rats of group 1 showed midzonal necrosis which extended into the centrilobular and periportal areas, leaving a narrow ring of parenchymal cells surrounding the central veins and portal tracts. The surviving cells were severely vacuolated, less basophilic than normal and PAS negative and often exhibited cytoplasmic eosinophilic globular hyaline. Kupffer cells in general were prominent and more numerous. Bile-duct proliferation was absent, but the epithelial cells were plump and prominent.

Male rats given vitamin A supplements (group 2) also showed liver damage. This was principally periportal in distribution, but often spread irregularly into adjacent midzones. Spotty and focal cell necrosis and fatty change were observed in the areas of surviving parenchymal cells, which showed a loss of cytoplasmic basophilia and eosinophilic degeneration. In three animals, glycogen was found only in the centrilobular areas, while in the remaining three it had disappeared from entire lobules. Prominence of the bile-duct epithelium and a variable degree of Kupffer-cell proliferation were also found in these animals, as in the rats of the other group.

The histopathological changes found in the male rats were thus similar in type in the two groups, but differed considerably in magnitude and distribution.

Liver damage found in the female rats was minimal and almost identical between the groups. The only changes observed were a mild loss of cytoplasmic basophilia, the presence of cytoplasmic eosinophilic globular hyaline in a few periportal hepatocytes and plumpness of the bile-duct epithelium. Liver glycogen was depleted, however, in the liver cells of the periportal zones.

Discussion

The nature of the interaction between aflatoxin and vitamin A is not yet clear, but observations made in the present study are important in the context of the wide prevalence of vitamin A deficiency in children in developing countries (Arroyave, Wilson, Mendez, Behar & Scrimshaw, 1961; Reddy & Srikantia, 1966). Experimentally induced protein-calorie malnutrition has been reported to increase the susceptibility of animals to aflatoxins (Madhavan & Gopalan, 1965; Madhavan *et al.* 1967), and vitamin A deficiency is yet another situation to be taken into account before safe limits are prescribed for this toxin in the human diet.

The aflatoxin-induced liver damage in male rats given the vitamin supplement (group 2) involved the periportal parenchyma, as was reported earlier (Butler, 1970). On the other hand, vitamin A-deficient male rats (group 1) mainly exhibited midzonal lesions, which extended, however, towards the peripheral and centrilobular zones. Thus a chronic deficiency of vitamin A apparently alters the distribution of demonstrable liver damage. The manner in which this is brought about requires elucidation.

Although hepatic stores of vitamin A were depleted to a similar extent in the males and females of group 1, hardly any liver injury was observed in the female rats. A relative resistance to aflatoxin in female rats has already been reported (Butler, 1970). In relation to the LD_{50} reported for female rats (Butler, 1970), the dose of aflatoxin used in the present study was very small.

Acknowledgements—The authors wish to express their thanks to Mr. A. Nadamuni Naidu for his help with

the statistical analysis of the data and Dr. K. Seetharam Bhat for his help with vitamin A determinations. They also acknowledge the technical assistance of Mr. N. K. Sreedharan and Mr. M. Purushotham.

REFERENCES

Allcroft, Ruth & Lewis, Gwyneth (1963). Groundnut toxicity in cattle: Experimental poisoning of calves and a report on clincial effects on older cattle. *Vet. Rec.* **75,** 487.

Arroyave, G., Wilson, D., Mendez, B. M. & Scrimshaw, N. S. (1961). Serum and liver vitamin A and lipids in children with severe protein malnutrition. *Am. J. clin. Nutr.* **9,** 180.

Butler, W. H. (1970). Liver injury induced by aflatoxin. In *Progress in Liver Diseases.* Vol. 3. Edited by H. Popper and F. Schaffner. p. 408. Grune and Stratton, New York.

Carnaghan, R. B. A., Lewis, G., Patterson, D. S. P. & Allcroft, Ruth (1966). Biochemical and pathological aspects of groundnut poisoning in chickens. *Path. vet.* **3,** 601.

Embree, N. D., Ames, S. R., Lehman, R. W. & Harris, P. L. (1957). Determination of vitamin A. In *Methods of Biochemical Analysis.* Vol. 4. Edited by D. Glick. p. 92. Interscience Publishers, Inc., New York.

Hamilton, P. B., Hsi-Tang Tung, Harris, J. R., Gainer, J. H. & Donaldson, W. E. (1972). The effect of dietary fat on aflatoxicosis in turkeys. *Poult. Sci.* **51,** 165.

Keyl, A. C., Booth, A. N., Masri, M. S., Gumbmann, M. R. & Gagne, W. E. (1970). Chronic effects of aflatoxin in farm animal feeding studies. In Proceedings of the First U.S.–Japan Conference on Toxic Micro-organisms, Honolulu, Hawaii. Edited by M. Herzberg. p. 72. US Department of the Interior and UJNR Panels on Toxic Micro-organisms, Washington, D.C.

Madhavan, T. V. & Gopalan, C. (1965). Effect of dietary protein on aflatoxin liver injury in weanling rats. *Archs Path.* **80,** 123.

Madhavan, T. V., Rao, K. S. & Tulpule, P. G. (1967). Effect of dietary protein level on susceptibility of monkeys to aflatoxin liver injury. *Indian J. med. Res.* **53,** 984.

Newberne, P. M. & Rogers, A. R. (1972). Vitamin A, liver and colon carcinoma in rats fed low levels of aflatoxin. *Toxic. appl. Pharmac.* **22,** 280.

Reddy, V. & Srikantia, S. G. (1966). Serum vitamin A in kwashiorkor. *Am. J. clin. Nutr.* **18,** 105.

Rogers, Adrianne E. & Newberne, P. M. (1971). Diet and aflatoxin B_1 toxicity in rats. *Toxic. appl. Pharmac.* **20,** 113.

Smith, J. W., Hill, C. H. & Hamilton, P. B. (1971). The effect of dietary modifications on aflatoxicosis in the broiler chicken. *Poult. Sci.* **50,** 768.

Fd Cosmet. Toxicol. Vol. 11, pp. 471–476. Pergamon Press 1973. Printed in Great Britain

Polychlorinated Biphenyls: Evidence of Transplacental Passage in the Sherman Rat

AUGUST CURLEY, V. W. BURSE and MARY E. GRIM

United States Environmental Protection Agency, Chamblee Toxicology Laboratory, 4770 Buford Highway, Chamblee, Georgia 30341, USA

(*Received 21 October 1972*)

Summary—The polychlorinated biphenyl (PCB), Aroclor 1254, was given orally in peanut oil to pregnant Sherman rats once daily from day 7 to day 15 of organogenesis. Dose levels were 0, 10 and 50 mg/kg/day. No statistical difference was found between control and dosed groups with respect to the total weight of litters, the percentage of pups born dead or the survival rate to weaning. Residue levels of PCB-derived material were measured in the foetuses, in the milk ingested by sucklings and in tissues of weanlings by electron-capture gas–liquid chromatography. Liver enlargement was observed in weanlings from dosed rats, but not in those from controls. There was a significant increase in the relative liver weights of weanlings from dosed rats compared with those from controls and the livers of most of the exposed weanlings contained enlarged hepatocytes, accompanied, in some cases, by cytoplasmic vacuolization and bile-duct proliferation, particularly in the group given the higher dosage.

Introduction

A previous study of oral dosing and dietary ingestion of Aroclor 1254® in Sherman rats demonstrated a wide distribution of material derived from chlorinated biphenyls (PCBs) in all tissues and excreta (Curley, Burse, Grim, Jennings & Linder, 1971). Other researchers have shown that some PCBs can induce microsomal-enzyme activity (Risebrough, Rieche, Peakall, Herman & Kirven, 1968) and cause porphyria (Vos & Koeman, 1970). The recent and persistent reports of PCBs in birds (Bagley, Reichel & Cromartie, 1970), fish (Risebrough *et al.* 1968) and man (Biros, Walker & Medbery, 1970) suggest that these compounds are as ubiquitous in the environment as *p,p'*-DDE.

Concentrations of *p,p'*-DDE, *p,p'*-DDT and other chlorinated hydrocarbons have been determined in human placentas and cord blood (O'Leary, Davies & Edmundson, 1970; Rappolt & Hale, 1968). The distribution of [^{14}C]DDT and dieldrin in pregnant mice (Backstrom, Hansson & Ullberg, 1965) and observable effects on the offspring resulting from the feeding of mirex to pregnant rats (Gaines & Kimbrough, 1970) all substantiate that transplacental passage of pesticides can occur.

The recent interest in PCBs and the transplacental movement exhibited by lipoid-soluble chlorinated compounds prompted this study. This paper reports the results of oral administration of Aroclor 1254 to pregnant rats and its subsequent detection in foetuses, weanlings and milk.

®Aroclor is a registered trade-name of Monsanto Co., St. Louis, USA.

Experimental

Three groups of female 90-day-old Sherman rats (mean body weight 254 g) were pair-mated with male rats of the same age. A vaginal smear from each female was examined microscopically each day until insemination was verified. Then male rats were removed. All rats were maintained on a diet of plain chow and water.

Considering the day of insemination as day 0, rats were given a single oral dose of Aroclor 1254 in peanut oil once a day on days 7–15 (inclusive) of organogenesis. The levels of dosage were 0, 10 and 50 mg/kg for groups 1, 2 and 3, respectively.

On day 20 of pregnancy, foetuses were taken by Caesarean section from three rats in each treated group and from two control rats. Foetuses from each dam were divided into two groups and analysed for PCB content as duplicate samples.

Ten dams in each group were allowed to deliver spontaneously. The litters were weighed, only the live rats being weighed in each group. When the pups were 5 days old, six mothers from each dosed group and three from the control group were removed from their offspring for several hours and then returned to their respective litters for 1 hr of nursing. Stomach content (milk) of pups was removed and pooled for each respective litter.

The litters of three mothers in each group were allowed to survive until weaning (21 days old). One male and one female weanling from each mother were weighed and sacrificed and the liver, brain and kidney were taken for analysis. The livers were weighed and sections of the liver from the other weanlings were examined microscopically.

Combined foetuses weighing 20·0 g were extracted with 200 ml hexane in an Omni-mixer for 5 min and filtered through a glass-wool plug into a 250 ml graduated cylinder. An aliquot was taken, the equivalent of 1·0 g. Milk (1·0 g) was homogenzied in 10 ml hexane. The extraction of other tissues including liver, kidney and brain, and the details of sample preparation, clean-up and analysis by electron-capture gas–liquid chromatography have been described previously (Curley *et al.* 1971).

Results

For animals subjected to Caesarean section on day 20, the control group averaged nine foetuses/dam while the dosed groups averaged ten foetuses/dam. All foetuses were normal upon gross examination. Of the dams allowed to deliver spontaneously, group 1 averaged 11·8 pups and 67·6 g/litter with none born dead, group 2 averaged 12·6 pups and 72·7 g/litter with one born dead and group 3 averaged 12·9 pups and 67·3 g/litter with four born dead.

Table 1 shows the concentrations of PCB-derived components found in foetuses taken by Caesarean section on day 20 of pregnancy. Measurable quantities of PCB-derived components were found in all samples except controls.

Although a fivefold difference in dosage level existed between group 2 given 10 mg/kg daily for 9 days and group 3 given 50 mg/kg daily for 9 days during organogenesis, the difference between the mean concentration of PCB in foetuses was only twofold. Residue levels in group 2 differed significantly ($P < 0{\cdot}001$) from those in group 3.

Table 2 shows the concentrations of PCB-derived components found in milk samples taken from suckling rats 11 days after the last dose was administered to the mother. PCB-derived components were found in all the milk samples analysed except those from the controls. There was a statistically significant difference ($P < 0{\cdot}001$) between groups 2 and 3. The mean concentrations of PCB in milk for the two treated groups showed a threefold difference for a fivefold difference in dosage.

Table 1. *Concentrations of PCB-derived components found in foetuses from rats given an oral dose of Aroclor 1254 on days 7–15 of pregnancy*

PCB dose (mg/kg/day)	No. of adult females	Mean body weight (g)	Level of PCB-derived components in foetuses (μg/g)*
0	2	253	< 0·12
10	3	258	0·63 ± 0·06 (0·40–0·78)
50	3	252	1·38 ± 0·06 (1·20–1·55)

*The values for the two dosed groups are means ± SEM for six determinations, with the range given in parenthesis.

Table 2. *Concentrations of PCB-derived components found in litter-pooled milk taken from suckling rats born to dams given an oral dose of Aroclor 1254 on days 7–15 of pregnancy*

PCB dose (mg/kg/day)	No. of adult females	Mean no. of pups/pooled sample	Level of PCB-derived components in the milk (μg/g)†
0	3	12	< 0·75
10	6	12·5	20·60 ± 1·59 (16·48–24·90)
50	6	12·3	66·34 ± 8·36* (45·80–100·29)

†The values for the two dosed groups are means ± SEM for six pooled samples, with the range given in parenthesis. The value marked with an asterisk differs significantly (Student's t test) from that for the other dosed group: *$P < 0·001$.

The average survival rate to weaning was 93·5, 96·0 and 90·0% for groups 1, 2 and 3, respectively. Table 3 summarizes the mean concentrations of PCB-derived components found in selected tissue in 21-day-old weanlings 27 days after the last dose was administered to the mother. Statistically, only one tissue, the kidney, showed a significant difference ($P < 0·001$) between male and female and this was at the higher dosage level.

The liver weights expressed as a precentage of body weights and the results of the microscopic examination are given in Table 4. There was a significant increase in the relative liver weights of dosed groups compared with those of controls.

The hepatocytes were enlarged in the majority of exposed rats. Vacuolization of the cytoplasm and proliferation of the bile ducts were seen in some rats. These morphological changes in the liver were more pronounced at the higher dosage.

Discussion

Components derived from Aroclor 1254 were found in the tissues of the foetuses taken from dosed mothers, but none were found in the control foetuses. This shows that transplacental passage of Aroclor occurs in rats. When 50 mg Aroclor/kg/day was given, only

Table 3. *Mean concentrations of PCB-derived components found in 21-day-old weanling rats from dams given an oral dose of Aroclor 1254 on days 7–15 of pregnancy*

PCB dose (mg/kg/day)	Levels of PCB-derived components (μg/g) in						Mean body weight of weanlings (g)	
	Brain		Liver		Kidney			
	Males	Females	Males	Females	Males	Females	Males	Females
0	< 0·74	< 0·94	< 0·19	< 0·22	< 0·36	< 0·30	42·4	38·5
10	2·16 ± 0·10	1·97 ± 0·43	3·35 ± 0·39	5·08 ± 1·64	1·69 ± 0·38	2·20 ± 0·24	34·8	33·4
50	7·69 ± 1·37	7·04 ± 1·91	18·24 ± 4·00	16·55 ± 2·79	6·99 ± 0·27*	2·92 ± 0·59	33·8	32·0

Values for tissue levels in the dosed group are means ± SEM for samples from three weanlings. That marked with an asterisk differs significantly (Student's *t* test) from the corresponding value for the other sex: *$P < 0·001$. The *t* test showed no significant differences between the body weight in weanlings from the control and dosed groups.

Table 4. *Relative liver weights and morphology of livers from 21-day-old weanlings from dams given an oral dose of Aroclor 1254 on days 7–15 of pregnancy*

PCB dose (mg/kg/day)	Mean liver weight (% of body weight)		Liver morphology: Light microscopic findings in rats of both sexes
	Males	Females	
0	3·8 ± 0·12	3·7 ± 0·06	Normal
10	4·8 ± 0·12**	5·1 ± 0·09*	Slightly enlarged and vacuolated hepatocytes in 15/21 rats
50	5·9 ± 0·13*	5·8 ± 0·06*	Slightly enlarged hepatocytes in 15/20 rats; vacuolization of the cytoplasm of the liver cells in 10/20 rats; bile-duct proliferation surrounded by fibrosis in 5/20 rats

Values are means ± SEM for three weanlings of either sex and those marked with asterisks differ significantly (Student's *t* test) from the control values: $*P < 0{\cdot}001$; $**P < 0{\cdot}005$.

twice as much PCB was found in the foetus as when 10 mg/kg/day was given. It is possible that distribution of the PCBs between the dam and the foetus is not uniform and that a partial barrier exists. This needs further investigation.

The PCB levels observed in the weanling rats resulted at least in part from nursing. The milk concentration of PCBs substantiates this conclusion. Also, the morphological changes observed in the liver, due to PCBs, most probably result from the PCB levels in the milk. An indication of the difference contaminated milk can make was shown for Mirex by Gaines & Kimbrough (1970). Sucklings exposed to mirex-contaminated milk developed cataracts, yet most sucklings raised on mirex-free milk did not, although they had been exposed *in utero* to this compound.

A statistical comparison of the control and dosed groups, with respect to the total weight of litters and the percentage of foetuses born dead, showed no significant difference.

Although the changes in liver morphology were more consistent at the higher dose, the overall effects of Aroclor ingestion on the liver parallel some findings previously observed (Kimbrough, Linder & Gaines, 1972). Rat livers were not all affected equally by the same dose. This has been observed in our laboratory before in PCB studies. It may be due, in part, to variations among individual animals and/or to impurities that may be constituents of the Aroclor preparations (Vos, Koeman, van der Maas, ten Noever de Brauw & de Vos, 1970).

Acknowledgements—The authors wish to thank Dr. R. D. Kimbrough for her interpretation of liver morphology.

REFERENCES

Bäckström, J., Hansson, E. & Ullberg, S. (1965). Distribution of C^{14}-DDT and C^{14}-dieldrin in pregnant mice determined by whole-body autoradiography. *Toxic. appl. Pharmac.* **7**, 90.

Bagley, G. E., Reichel, W. L. & Cromartie, E. (1970). Identification of polychlorinated biphenyls in two bald eagles by combined gas–liquid chromatography-mass spectrometry. *J. Ass. off. analyt. Chem.* **53**, 251.

Biros, F. J., Walker, Annita C. & Medbery, Angela (1970). Polychlorinated biphenyls in human adipose tissue. *Bull. env. contam. & Toxicol. (U.S.)* **5**, 317.

Curley, August, Burse, V. W., Grim, Mary E., Jennings, R. W. & Linder, R. E. (1971). Polychlorinated biphenyls: Distribution and storage in body fluids and tissues of Sherman rats. *Envir. Res.* **4**, 481.

Gaines, T. B. & Kimbrough, R. D. (1970). Oral toxicity of mirex in adult and suckling rats. With notes on the ultrastructure of liver changes. *Archs envir. Hlth* **21**, 7.

Kimbrough, R. D., Linder, R. E. & Gaines, T. B. (1972). Morphological changes in livers of rats fed polychlorinated biphenyls. *Archs envir. Hlth* **25,** 354.

O'Leary, J. A., Davies, J. E. & Edmundson, W. F. (1970). Transplacental passage of pesticides. *Am. J. Obstet. Gynec.* **107,** 65.

Rappolt, R. T. & Hale, W. E. (1968). p,p′-DDE and p,p′-DDT residues in human placentas, cords, and adipose tissue. *Clin. Toxicol.* **1,** 57.

Risebrough, R. W., Rieche, P., Peakall, D. B., Herman, S. G. & Kirven, M. N. (1968). Polychlorinated biphenyls in the global ecosystem. *Nature, Lond.* **220,** 1098.

Vos, J. G. & Koeman, J. H. (1970). Comparative toxicologic study with polychlorinated biphenyls in chickens with special reference to porphyria, edema formation, liver necrosis, and tissue residues. *Toxic. appl. Pharmac.* **17,** 656.

Vos, J. G., Koeman, J. H., van der Maas, H. L., ten Noever de Brauw, M. C. & de Vos, R. H. (1970). Identification and toxicological evaluation of chlorinated dibenzofuran and chlorinated naphthalene in two commercial polychlorinated biphenyls. *Fd Cosmet. Toxicol.* **8,** 625.

Fd Cosmet. Toxicol. Vol. 11, pp. 477–495. Pergamon Press 1973. Printed in Great Britain

MONOGRAPHS

Monographs on Fragrance Raw Materials*

D. L. J. OPDYKE

Research Institute for Fragrance Materials, Inc., P.O. Box 1152, Englewood Cliffs, New Jersey 07632, USA

(*Received 7 January 1973*)

ALDEHYDE C-10

Synonyms: Decyl aldehyde; capraldehyde; decanal.
Structure: $CH_3 \cdot [CH_2]_8 \cdot CHO$.
Description and physical properties: EOA Spec. no. 26.
Occurrence: Decyl aldehyde is the most widely occurring of all the fatty aldehydes. Over 50 sources including citrus oils, citronella and lemongrass contain this aldehyde (Gildemeister & Hoffman, 1963).
Preparation: By catalytic oxidation of decyl alcohol.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 25,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·025	0·06
Maximum	0·1	0·09	0·01	0·1

Analytical data: Gas chromatogram, RIFM no 70-30; infra-red curve, RIFM no. 70-30.

Status

Aldehyde C-10 was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1972) listed aldehyde C-10 (decanal), giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on aldehyde C-10.

Biological data

Acute toxicity. The acute oral LD_{50} value was reported as > 33·32 g/kg in the rat, and as > 41·75 g/kg in the mouse (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). Smyth, Carpenter, Weil, Pozzani & Striegel (1962) reported a single-dose oral LD_{50} in rats as 3·73 ml/kg. The acute dermal LD_{50} for rabbits is given as 5·04 ml/kg (Smyth *et al.* 1962).

Human testing. A standard patch test using full strength aldehyde C-10 for 24 hours produced no reactions in any of 28 subjects (Katz, 1946).

Metabolism. See monograph on aldehyde C-8†.

*The first set of these monographs was published in *Food and Cosmetics Toxicology* (1973, **11**, 95).
†*Food and Cosmetics Toxicology* 1973, **11**, 113.

References

Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VI, Series 1, no. 98, p. 42. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2362. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 231. National Academy of Sciences–National Research Council, Washington, D.C.

Gildemeister, E. u. Hoffman, F. (1963). *Die Ätherischen Öle*. Vol. IIIc. p. 28. Akademie Verlag, Berlin.

Jenner, P. M., Hagan, E. C., Taylor, Jean M., Cook, E. L. & Fitzhugh, O. G. (1964). Food flavourings and compounds of related structure. I. Acute oral toxicity. *Fd Cosmet. Toxicol.* **2,** 327.

Katz, A. (1946). *Spice Mill* **69** (July), 46.

Smyth, H. F., Jr., Carpenter, C. P., Weil, C. S., Pozzani, U. C. & Striegel, Jean A. (1962). Range-finding toxicity data: List VI. *Am. ind. Hyg. Ass. J.* **23,** 95.

ALDEHYDE C-11 UNDECYLENIC

Synonyms: Undecenal; undecylenaldehyde; hendecenal; undecene-10-al.

Structure: $CH_2:CH\cdot[CH_2]_8\cdot CHO$.

Description and physical properties: EOA Spec. no. 84.

Occurrence: Apparently has not been reported to occur in nature.

Preparation: By oxidation of the corresponding alcohol or by reduction of the corresponding acid chloride.

Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·0025	0·04
Maximum	0·06	0·009	0·01	0·125

Analytical data: Gas chromatogram, RIFM no. 70-31; infra-red curve, RIFM no. 70-31.

Status

Aldehyde C-11, undecylenic was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1972) included aldehyde C-11, undecylenic (undec-10-enal) in the list of admissible artificial flavouring substances at a level of 0·2 ppm. The *Food Chemicals Codex* (1972) has a monograph on aldehyde C-11, undecylenic.

Biological data

Acute toxicity. Both the oral LD_{50} value in rats and the dermal LD_{50} value in rabbits exceeded 5 ml/kg (Hart, 1971).

Human testing. A maximization test (Kligman, 1966) carried out on 25 volunteers using a 1% concentration in petrolatum produced no reactions (Kligman, 1971).

References

Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VI, Series 1, no. 122, p. 44. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 3095. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex of the Committee on Food Protection. p. 846. National Academy of Sciences–National Research Council Washington, D.C.

Hart, E. R. (1971). Report to RIFM, 18 June.

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. (1971). Report to RIFM, 3 November.

ALDEHYDE C-11, UNDECYLIC

Synonyms: Undecanal; hendecanal.

Structure: $CH_3 \cdot [CH_2]_9 \cdot CHO$.

Description and physical properties: EOA Spec. no. 123.

Occurrence: Has been reported to occur in lemon and mandarin oils (Gildemeister & Hoffman, 1963).

Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·0025	0·04
Maximum	0·06	0·009	0·1	0·5

Analytical data: Gas chromatogram, RIFM no. 71-15; infra-red curve, RIFM no. 71-15.

Status

Aldehyde C-11, undecylic was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1972) included aldehyde C-11, undecylic (undecanal) in the list of admissible artificial substances at a level of 5 ppm. The *Food Chemicals Codex* (1972) has a monograph on aldehyde C-11, undecylic.

Biological data

Acute toxicity. Both the oral LD_{50} value in rats and the dermal LD_{50} value in rabbits exceeded 5 g/kg (Shelanski, 1971).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 5% in petrolatum and produced no reactions (Kligman, 1971).

References

Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VI, Series 1, no. 121, p. 44. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 3092. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 845. National Academy of Sciences–National Research Council, Washington D.C.

Gildemeister, E. u. Hoffman, F. (1963). *Die Ätherischen Öle*. Vol. IIIc. Akademie Verlag, Berlin.

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. (1971). Report to RIFM, 27 September.

Shelanski, M. V. (1971). Report to RIFM, 14 November.

ALDEHYDE C-12, LAURIC

Synonyms: Lauryl aldehyde; dodecanal; *n*-dodecylic aldehyde; duodecylic aldehyde.
Structure: $CH_3 \cdot [CH_2]_{10} \cdot CHO$.
Description and physical properties: EOA Spec. no. 51.
Occurrence: Reported to occur in pine-needle, lime, sweet-orange and a dozen other essential oils (Gildemeister & Hoffman, 1963).
Preparation: By catalytic oxidation of the corresponding alcohol.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·0025	0·04
Maximum	0·06	0·009	0·1	0·125

Status

Aldehyde C-12, lauric was classified GRAS by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1972) listed aldehyde C-12, lauric (dodecanal), giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on aldehyde C-12, lauric.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as 23·1 g/kg in the rat (Calandra, 1971). The acute dermal LD_{50} was reported to be > 2 g/kg in the rabbit (Calandra, 1971).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 1 % in petrolatum and produced no reactions (Kligman, 1970).

Metabolism. See monograph on aldehyde C-8*.

References

Calandra, J. C. (1971). Report to RIFM, 12 April.
Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VI, Series 1, no. 99, p. 42. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2615. *Fd Technol., Champaign* **19** (2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 441. National Academy of Sciences–National Research Council, Washington, D.C.
Gildemeister, E. u. Hoffman, F. (1963). *Die Ätherischen Öle.* Vol. IIIc. p. 32. Akademie Verlag, Berlin.
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. (1970). Report to RIFM, 2 December.

Food and Cosmetics Toxicology* 1973, **11, 113.

ALDEHYDE C-12, MNA

Synonyms: Methyl n-nonyl acetaldehyde; 2-methyl undecanal.
Structure: $CH_3 \cdot [CH_2]_8 \cdot CH(CH_3) \cdot CHO$.
Description and physical properties: EOA Spec. no. 52.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By decomposition of the corresponding glycidic acid (Bedoukian, 1967).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 35,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·0025	0·04
Maximum	0·06	0·009	0·01	0·4

Analytical data: Gas chromatogram, RIFM no. 71-16; infra-red curve, RIFM no. 71-16.

Status

Aldehyde C-12, MNA was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1972) included aldehyde C-12, MNA (2-methyl undecanal) in the list of temporarily admissible artificial flavouring substances pending further studies. The *Food Chemicals Codex* (1972) has a monograph on aldehyde C-12, MNA.

Biological data

Acute toxicity. The acute oral LD_{50} value (RIFM sample no. 71-16) was reported as > 5 g/kg in the rat (Owen, 1971). The acute dermal LD_{50} for sample no. 71-16 was reported to be > 10 g/kg in the rabbit (Owen, 1971).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no reactions (Kligman, 1971).

Metabolism. See monograph on aldehyde C-8*.

References

Bedoukian, P. Z. (1967). *Perfumery and Flavouring Synthetics.* 2nd ed., p. 21. Elsevier Publishing Co., New York.
Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VII, Series 2, no. 2010, p. 80. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2749. *Fd Technol., Champaign* **19** (2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 536. National Academy of Sciences–National Research Council, Washington, D.C.
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. (1971). Report to RIFM, 17 June.
Owen, G. (1971). Report to RIFM, 28 June and 1 July.

Food and Cosmetics Toxicology* 1973, **11, 113.

ALDEHYDE C-14, MYRISTIC

Synonyms: Myristic aldehyde; tetradecanal.
Structure: $CH_3 \cdot [CH_2]_{12} \cdot CHO$.
Description and physical properties: Givaudan Index (1961).
Occurrence: Found in several essential oils.
Preparation: By catalytic oxidation of myristic alcohol.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

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

Analytical data: Gas chromatogram, RIFM no. 71-17; infra-red curve, RIFM no. 71-17.

Status

Aldehyde C-14, myristic was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1972) has included aldehyde C-14, myristic (tetradecanal) in the list of admissible artificial flavouring substances at a level of 3 ppm.

Biological data

Acute toxicity. The acute oral LD_{50} value (RIFM sample no. 71-17) was reported as > 5·0 g/kg in the rat (Lynch, 1971). The acute dermal LD_{50} for sample no. 71-17 was reported to be > 10 g/kg (Lynch, 1971).

Human testing: A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 1 % in petrolatum and produced no reactions (Kligman, 1971).

Metabolism: See monograph on aldehyde C-8*.

References

Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VI, Series 1, no. 118, p. 44. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels, No. 2763. *Fd Technol., Champaign* **19** (2), part 2, 155.
Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery*. 2nd ed., p. 40. Givaudan-Delawanna, Inc., New York.
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. (1971). Report to RIFM, 17 June.
Lynch, T. A. (1971). Report to RIFM, 16 June.

Food and Cosmetics Toxicology* 1973, **11, 113.

ALLYL CAPROATE

Synonyms: Allyl hexanoate; 2-propenyl hexanoate.
Structure: $CH_2:CH\cdot CH_2\cdot OOC[CH_2]_4\cdot CH_3$.
Description and physical properties: EOA Spec. no. 122.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By direct esterification of allyl alcohol with caproic acid.
Uses: In public use since the early 1920s. Use in fragrances in the USA amounts to less than 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·0025	0·0003	0·0013	0·05
Maximum	0·015	0·0015	0·005	0·125

Analytical data: Gas chromatogram, RIFM no. 71-20; infra-red curve, RIFM no. 71-20.

Status

Allyl caproate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The *Food Chemicals Codex* (1972) has a monograph on allyl caproate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was 218 mg/kg and in guinea-pigs 280 mg/kg (Taylor, Jenner & Jones, 1964). The acute dermal LD_{50} for sample no. 71-20 was reported as 0·3 (0·2–0·6) ml/kg in the rabbit (Shelanski, 1971).

Chronic toxicity. In an FDA feeding study, 2500 ppm fed to rats in the diet for 1 yr produced no effects (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). In another feeding study, 0·1–0·5% fed to rats in the diet for 2 yr suppressed growth (Bär & Griepentrog, 1967).

Human testing. A maximization test (Kligman, 1966), carried out on 25 volunteers using a 4% concentration in petrolatum, produced no reactions (Kligman, 1971).

Metabolism. Clapp, Kaye & Young (1969) have reported on the metabolism of allyl compounds in the rat. These compounds react with reduced glutathione in the liver and the resultant product, after hydrolysis and *N*-acetylation gives rise to the mercapturic acid, which is readily excreted in the urine.

References

Bär, F. u. Griepentrog, F. (1967). Die Situation in der gesundheitlichen Beurteilung der Aromatisierungsmittel für Lebensmittel. *Medizin Ernähr.* **8,** 244.

Clapp, J. J., Kay, C. M. & Young, L. (1969). Observations on the metabolism of allyl compounds in the rat. *Biochem. J.* **114,** 6P.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2032. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 29. National Academy of Sciences–National Research Council, Washington, D.C.

Hagan, E. C., Hansen, W. H., Fitzhugh, O. G., Jenner, P. M., Jones, W. I., Taylor, Jean M., Long, Eleanor L., Nelson, A. A. & Brouwer, J. B. (1967). Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Fd Cosmet. Toxicol.* **5,** 141.

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. (1971). Report to RIFM, 27 September.
Shelanski, M. V. (1971). Report to RIFM, 26 November.
Taylor, Jean M., Jenner, P. M. & Jones, W. I. (1964). A comparison of the toxicity of some allyl, propenyl, and propyl compounds in the rat. *Toxic. appl. Pharmac.* **6**, 378.

ALLYL CYCLOHEXYLPROPIONATE

Synonyms: Allyl 3-cyclopropionate; allyl hexahydrophenylpropionate.
Structure: $CH_2:CH\cdot CH_2\cdot OCOCH_2\cdot CH_2\cdot C_6H_{11}$.
Description and physical properties: EOA Spec. no. 254.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By esterification of allyl alcohol with cyclohexylpropionic acid.
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 30,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·0025	0·09
Maximum	0·045	0·009	0·015	0·375

Analytical data: Gas chromatogram, RIFM no. 71-21; infra-red curve, RIFM no. 71-21.

Status

Allyl cyclohexylpropionate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1972) included allyl cyclohexylpropionate in the list of temporarily admissible artificial flavouring substances at a limit of 10 ppm. The *Food Chemicals Codex* (1972) has a monograph on allyl cyclohexylpropionate.

Biological data

Acute toxicity. The acute oral LD_{50} has been reported as 585 mg/kg in the rat (Bär & Griepentrog, 1967).

Chronic toxicity. In feeding studies, 2500 ppm fed to rats in the diet for 52 wk (Bär & Griepentrog, 1967) produced no effects.

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no reactions (Kligman, 1971).

Metabolism. Allyl compounds are metabolized to mercapturic acid which is excreted in the urine (Clapp, Kaye & Young, 1969). Cyclohexylpropionic acid is aromatized to benzoic acid and excreted as hippuric acid in the urine. Substituted cyclohexylcarboxylic acids are either excreted unchanged or completely oxidized (Williams, 1959).

References

Bär, F. u. Griepentrog, F. (1967). Die Situation in der gesundheitlichen Beurteilung der Aromatisierungsmittel für Lebensmittel. *Medizin Ernähr.* **8**, 244.

Clapp, J. J., Kaye, C. M. & Young, L. (1969). Observations on the metabolism of allyl compounds in the rat. *Biochem. J.* **114**, 6P.

Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VII, Series 2, no. 2223, p. 91. Strasbourg.

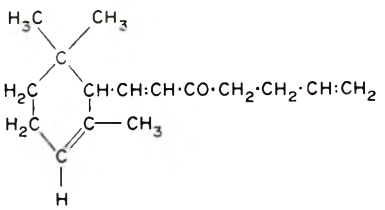
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2026. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 28. National Academy of Sciences–National Research Council, Washington, D.C.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screeening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1971). Report to RIFM, 3 November.
Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed. p. 119. Chapman & Hall, Ltd., London.

ALLYL-α-IONONE

Synonyms: Allyl ionone.
Structure:



Description and physical properties: Food Chemicals Codex (1972).
Occurrence: Apparently has not been reported to occur in nature (*Givaudan Index*, 1961).
Preparation: By condensation with allyl acetone from citral, followed by cyclization (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·0005	0·0025	0·09
Maximum	0·03	0·0045	0·02	1·0

Analytical data: Gas chromatogram, RIFM no. 71-22; infra-red curve, RIFM no. 71-22.

Status

Allyl-α-ionone was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1972) included allyl-α-ionone in the list of temporarily admissible artificial flavouring substances pending further studies. *The Food Chemicals Codex* (1972) has a monograph on allyl-α-ionone.

Biological data

Acute toxicity. The acute dermal LD_{50} was reported to be > 5 g/kg in the rabbit (Shelanski, 1971).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no reactions (Kligman, 1972).

Metabolism. α-Ionones are metabolized by ring hydroxylation at the carbon atom alpha to the ring double bond to give 5-hydroxy-α-ionone (Williams, 1959). Allyl compounds are reported to form mercapturic acids (Clapp, Kaye & Young, 1969).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals* (*Aroma Chemicals*). Vol. 1, p. 86. S. Arctander, Montclair, New Jersey.

Clapp, J. J., Kaye, C. M. & Young, L. (1969). Observations on the metabolism of allyl compounds in the rat. *Biochem. J.* **114,** 6P.

Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VII, Series 2, no. 2040, p. 143. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2033. *Fd Technol., Champaign* **19** (2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 30. National Academy of Sciences–National Research Council, Washington, D.C.

Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery*. 2nd ed., p. 94. Givaudan-Delawanna, Inc., New York.

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. (1972). Report to RIFM, 21 February.

Shelanski, M. V. (1971). Report to RIFM, 14 November.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds*. 2nd ed., p. 539. Chapman & Hall, Ltd., London.

AMYL BENZOATE

Synonyms: Isoamyl benzoate; 3-methyl-1-butyl benzoate.
Structure: $(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH_2 \cdot OCOC_6H_5$.
Description and physical properties: Givaudan Index (1961).
Occurrence: Reported to occur in cherry oil (Stinson, Dooley, Filipic & Hills, 1969).
Preparation: By esterification of isoamyl alcohol with benzoic acid.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·18
Maximum	0·09	0·009	0·03	0·6

Analytical data: Gas chromatogram, RIFM no. 71-24; infra-red curve, RIFM no. 71-24.

Status

Amyl benzoate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1972) has listed amyl benzoate (isoamyl benzoate) giving an ADI of 5 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} value (RIFM sample no. 71-24) was reported as 6·33 g/kg in the rat (Weir, 1971). The acute dermal LD_{50} for sample no. 71-24 was reported to be > 5 g/kg in the rabbit (Weir, 1971).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 6% in petrolatum and produced no reactions (Kligman, 1972).

Metabolism. Benzoic acid is metabolized in the mammalian body after conjugation with glycine to form hippuric acid and benzoylglucuronic acid, which are excreted in the urine (Williams, 1959).

References

Council of Europe (1972). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Section VI, Series 1, no. 562, p. 68. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2058. *Fd Technol., Champaign* **19** (2), part 2, 155.

Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery.* 2nd ed., p. 47. Givaudan-Delawanna, Inc., New York.

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. (1972). Report to RIFM, 3 November.

Stinson, E. E., Dooley, C. J., Filipic, V. J. & Hills, C. H. (1969). *J. Fd Sci.* **34,** 544.

Weir, R. J. (1971). Report to RIFM, 25 August.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed., p. 349. Chapman & Hall Ltd., London.

Fd Cosmet. Toxicol. Vol. 11, pp. 497–498. Pergamon Press 1973. Printed in Great Britain

Review Section

REVIEWS OF RECENT PUBLICATIONS

Specifications for the Identity and Purity of Some Enzymes and Certain Other Substances. Joint FAO/WHO Expert Committee on Food Additives. WHO/Food Add./72.2; *F.A.O. Nutr. Mtg Rep. Ser.* no. 50B, Rome, 1972. pp. 174. £0.90.

This document provides specifications for most of the substances discussed at the Fifteenth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (*Cited in F.C.T.* 1973, **11,** 296). Its publication has been eagerly awaited, since the validity of the evaluations already published is largely dependent on the adequacy of the specifications. This is particularly so in the case of the 23 enzyme preparations—six derived from animals, four from plants and 13 from microbial sources—that are covered. It is somewhat disappointing, therefore, to find that the general specification for these materials describes acceptable enzyme preparations from microbial sources as those "produced by methods and under suitable culture conditions which ensure a controlled fermentation and prevent the introduction of micro-organisms which could be the source of toxic materials and other undesirable substances", without any further details. An encouragingly low limit, of 5 μg/kg, has been set for aflatoxin, but the establishment of tolerances for other mycotoxins has been postponed until suitable detection methods are available. Other prescribed maximum limits are for arsenic (3 mg/kg), lead (10 mg/kg) and heavy metals (50 mg/kg calculated as lead), while the absence of Salmonella, Pseudomonas and antibiotic activity and a limit of 30/g on coliforms are also specified. This specification for enzyme preparations also deals with additives and ingredients, although again only in a very general manner, and with hygiene and enzyme activity.

Other specifications cover caramel colourings (the specification on ammonia-process caramels being only tentative), microcrystalline cellulose, sodium and calcium stearoyl lactylates, esters of glycerol and thermally oxidized soya-bean fatty acids, hexamethylenetetramine and chemically treated starches, the last-named being a replacement for a specification drawn up in 1970 (Joint FAO/WHO Expert Committee on Food Additives, WHO/Food Add./70.37; *F.A.O. Nutr. Mtg Rep. Ser.* no. 46B). An annex gives methods for the examination of enzyme preparations and of caramel (including tests for ammoniacal nitrogen, 4-methylimidazole and colour intensity), calcium stearoyl lactylate (acid value, calcium content, ester value and total lactic acid), sodium stearoyl lactylate (sodium content), fatty acids not forming adducts with urea and chemically treated starches.

International Standards for Drinking-water. Third edition. World Health Organization, Geneva, 1971. pp. 70. £0.90.

Since 1963, when the second edition of this publication was issued, knowledge of the nature and effects of various contaminants has increased and techniques for their identification and determination have improved. The present volume is considerably shorter than previous editions, more than two-thirds of which were devoted to a detailed description of approved methods of water examination, which are now readily available elsewhere. The list of suggested subjects for research has also been omitted, and less space has been devoted to the evidence considered by the Committee in recommending limits for the concentrations of particular substances.

The publication deals with the bacteriological, virological, biological, radiological and physical and chemical examination of water supplies and with sampling frequency, collection, transport and storage. Maximum limits proposed for arsenic, cadmium, cyanide, lead, selenium, fluoride and radioactivity are now identical with those given in the analogous European standards (*Cited in F.C.T.* 1972, **10,** 228). However, limits for chromium and barium have been omitted, that for polynuclear aromatic hydrocarbons has been raised from 0·02 to 0·2 μg/litre and a new limit of 0·001 mg/litre for mercury has been based on levels found in natural water rather than on toxicological data. If polymeric coagulant aids, such as polyacrylamide, are used in the treatment of drinking-water, it is considered necessary to ensure that any toxic components, such as the monomer, are reduced to safe levels by insistence on an adequate specification for the chemicals used. For nitrates, a concentration greater than 45 mg/litre is now regarded as dangerous to infants from the point of view of methaemoglobinaemia, and this recommended limit may be reduced in future if it is found that the nitrate content of drinking-water contributes significantly to the hazard of nitrosamine formation *in vivo*.

There are many additional substances that may affect the acceptability of water, rather than its safety. These include substances causing discoloration, odours or tastes, suspended matter, solids, pH range, anionic detergents, mineral oil, phenolic compounds, total hardness, calcium, chloride, copper, iron, magnesium, manganese, sulphate and zinc, for each of which a highest desirable level and a maximum permissible level are given and a method of estimation is specified.

WHO hopes that the publication of this revised edition will stimulate further investigations into the provision of safe drinking-water to all communities, the function of water quality in maintaining public health and the improvement of treatment processes. It is recognized that future developments may make further revision necessary, and constructive criticism and suggestions based upon experience would be welcomed by WHO.

Fd Cosmet. Toxicol. Vol. 11, pp. 499–502. Pergamon Press 1973. Printed in Great Britain

BOOK REVIEWS

Fundamental Principles and Objectives of a Comparative Food Law. Vol. 4. Elements of Control and Sanction; General Conclusion of the Study; Suggested Outline of a Modern Food Law. By E. J. Bigwood and A. Gérard. S. Karger, Basel, 1971. pp. xiv+329. £9.60.

This volume marks the conclusion of an extensive study begun in 1965 by the Food Law Research Centre of the Institute of European Studies, Brussels University. Volumes 1, 2 and 3 presented an in-depth appraisal of the various types of food laws operating in 15 countries and of their application and interpretation. Volume 4, as its title indicates, gives the group's general conclusions and suggests the outline within which a modern food law could be constructed.

The purpose of food law harmonization is to prevent, in the future, the existence of discordant regulations that introduce obstacles to the free international circulation of food products and thus serve as non-tariff barriers. The findings of the study suggest that a proper outline for a modern system of food regulation, leading to an integrated, common and flexible food law to be recognized and adopted by all countries interested in the harmonization of the basic principles of their respective food regulations, must be sought in a system which takes a critical look at certain defined principles ranging from the recognition of the concept of acceptable hazard to the contents of food standards. The principal differences between the various national food laws are identified and analysed on a procedural, technical and scientific basis and suggestions are presented for changes that might minimize or eliminate these differences. This represents only part of the solution, however; such changes need to be co-ordinated into effective law, and this in itself presents certain difficulties. A case in point arises from the established use of negative or positive lists for food additives (based, respectively, on the principles of abuse or prohibition), the former being operative in some countries and the latter in others. As a solution to this problem, the group suggests the adoption of a mixed system of control based upon the division of additives into two categories, with those that occur naturally in the human body or elsewhere being controlled by a negative-list system and those that are produced synthetically being regulated by a positive-list system. A substance obtained synthetically but having a chemical composition identical to that of a natural substance would be subject to negative-list control.

The volume also discusses the organization of the research activities that must produce the scientific data essential as a basis for political and legal discussion and stresses the need to disassociate scientific preparation and research from political decision. To obviate any tendency of the experts from the various governments to take up nationalistic attitudes, an international institution could be entrusted with decision-making of a purely technical or scientific character. Such an institution should consist of experts selected by their governments, but not directly responsible to them. Transferring this concept to the level of the EEC, it is suggested that a permanent scientific body possessing freedom of action should determine the scientific content of draft directives. Co-ordination of the work of this body

with that undertaken by other international organizations, such as the Codex Alimentarius Commission and the Council of Europe, would be an essential adjunct.

An appendix to this volume presents a detailed appraisal of current thinking on food-packaging regulations in various European countries as well as Canada and the USA.

Residue Reviews. Residues of Pesticides and Other Foreign Chemicals in Foods and Feeds. Vol. 39. The Carbinole Acaricides: Chlorobenzilate and Chloropropylate. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1971. pp. vii+93. DM 49.50.

Close on the heels of the first Residue Reviews monograph on a single pesticide (*Cited in F.C.T.* 1972, **10,** 562) has come a similar volume, dealing this time with two closely related compounds, chlorobenzilate and chloropropylate. Like the previous monograph, it was written by the staff of CIBA-GEIGY Ltd., the company responsible for the development of the pesticides. The compounds are treated separately, because they were developed at different times and the respective experiments were therefore not conducted in parallel. For each a detailed account is given of toxicology in laboratory animals and wildlife, metabolism, efficacy and mode of action (which includes a welcome lack of effect on non-target organisms such as bees), residues and analytical methods. Finally, a useful summary is provided in English, French and German, followed by more than five pages of references relating to the two compounds.

Many of the above topics were reviewed in the FAO/WHO monographs on the two pesticides published in 1969 (*ibid* 1970, **8**, 184), but coverage has been expanded in the present volume, largely by the addition of unpublished data obtained by the manufacturers. The sections on toxicology have been enlarged to include information on dermal as well as oral toxicity, although the latter topic has been subject to a few omissions—notably of the increased incidence of chronic renal disease seen in a long-term feeding test on chloropropylate in rats and considered worthy of comment by FAO/WHO. However, this is but a small criticism of what would otherwise seem an exhaustive and painstaking treatment of the subject. This volume should be of value to anyone concerned with the use or legislative control of either pesticide, and it is to be hoped that more of such comprehensive monographs will be issued in the near future.

Side Effects of Drugs. Vol. VII. A Survey of Unwanted Effects of Drugs Reported in 1968–1971. Edited by L. Meyler and A. Herxheimer. Excerpta Medica, Amsterdam, 1972. pp. x+758. Dfl.136.00.

This is the seventh volume of a well known and valuable series, which was started in 1957. However, this volume differs from its predecessors in one important respect, in that each chapter contains brief notes summarizing previously published observations and thus provides a comprehensive background to the side effects reported during 1968–71, which are covered in greater detail in the usual way.

Each of the 47 chapters covers a group of drugs, and the authors of each chapter attempt a critical assessment of the raw data from which the contribution is compiled. In clinical practice, a decision that a patient's symptoms and/or changes in clinical chemistry are due to the drug he has been given may be very difficult, and some authors are more courageous than others in formulating opinions as to cause and effect.

The value of this volume is enhanced by reference to toxicity in animals and also to cases of accidental or deliberate overdosage. Consequently, while practising clinicians will comprise the largest group to benefit from this volume, peripheral groups working in forensic and other branches of toxicology will also find it an essential handbook. These latter groups in particular will appreciate the list of references appended to each chapter. A good general index and a helpful supplementary list of proprietary names with their chemical or generic equivalents are provided.

In summary, this volume may be recommended to all who need to know about the adverse effects which a wide range of chemical compounds may produce in man.

Intestinal Absorption of Metal Ions, Trace Elements and Radionuclides. Edited by S. C. Skoryna and D. Waldron-Edward. Pergamon Press, Oxford, 1971. pp. xv+431. £7.

Although this book is intended for "both clinician and basic scientist", much emphasis has been placed on the theoretical aspects and *in vitro* studies of metal-ion, trace-element and radionuclide transport. There is relatively little paramedical information on the intestinal absorption of these metals, in spite of the title of the book. The clinician, nutritionist or gastro-enterologist may, therefore, find this text heavy-going, and may well be somewhat disappointed by its contents. However, the basic scientist should find it a useful, if not invaluable, treatise. A number of eminent contributors to the book are experts in the field of intestinal absorption. Their articles are instantly recognizable from their well-known, well-documented contents and the authors' personal hypotheses, but there is a lack of new original data.

The editing of the book is not entirely satisfactory. A general and unbiased summary of all the articles would have met an obvious need and could have been used to iron out certain discrepancies between authors. Moreover there is a considerable amount of monotonous repetition. This is especially noticeable in the introductory paragraphs, where each article in turn may present a similar historical picture with the same appropriate references. Indeed, on looking through the Author Index, it is not unusual to find authors who have been quoted in the book twenty or more times.

The papers deal more than adequately with the transport of a number of metal ions, trace elements and radionuclides. In particular, the transport of sodium, potassium and calcium is discussed extensively, and that of magnesium, zinc, copper, cobalt, strontium, aluminium, radiocaesium and radioruthenium is also well covered. There are, however, notable omissions, such as selenium, which would be of interest to the nutritionist and clinician. Data on the absorption of the substances under investigation have been largely confined to *in vitro* studies of transport in intestinal preparations and mitochondria and even in plant cells. It is surprising that studies of uptake in bacteria were not included, as these would have been at least as relevant as those in plants. Several nutritional and paramedical points that are made could usefully have been developed further. Among these are the effects of dietary changes on the absorption of calcium, iron and zinc, and the role of alginates as therapeutic metal-binders in medicine. The effects of other factors, such as hormones, pH, bile salts and vascular changes, on metal-ion absorption are evaluated, however.

The general conclusion of the authors represented in the book is that there is still a great deal of research to be done on the subject of metal-ion and trace-element absorption. The

use of new isotope techniques is advocated as one possible approach to the problem. Studies on the absorption of strontium and radiocaesium and of heavy metals, including lead and mercury, are of the utmost importance in connexion with current questions of environmental pollution, and this book can assist our appreciation of the problem.

BOOKS RECEIVED FOR REVIEW

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Vol. 1. International Agency for Research on Cancer, Lyon, 1972. pp. 184. £0.40.

Epidemiology of DDT. Edited by J. E. Davies and W. F. Edmundson. Futura Publishing Company, New York, 1972. pp. ix+157. $14.95

Methods of Aflatoxin Analysis. By B. D. Jones. Tropical Products Institute, London, 1972. pp. v+58.

The Use of Drugs in Animal Feeds. Proceedings of a Symposium. National Academy of Sciences, Washington, 1969. pp. viii+407. $8.50.

Methods in Morbid Anatomy. By R. R. Wilson. Butterworths, London, 1972. pp. xi+156. £4.25.

Metallic Contaminants and Human Health. Edited by D. H. K. Lee. Academic Press, New York, 1972. pp. xvii+241. £3.25.

Health and Food. Edited by G. G. Birch, L. F. Green and L. G. Plaskett. Applied Science Publishers Ltd., London, 1972. pp. xi+224. £4.20.

Methylmercury. A Review of Health Hazards and Side Effects Associated With the Emission of Mercury Compounds Into Natural Systems. By G. Löfroth. Ecological Research Committee, Bulletin no. 4 (2nd ed.). Swedish Natural Science Research Council, 1970. pp. 59. 12 skr.

Health Hazards of the Human Environment. World Health Organization, Geneva, 1972. pp. 388. £4.40.

Current Topics in Biochemistry. National Institute of Health Lectures in Biomedical Sciences. Edited by C. B. Anfinsen, R. F. Goldberger and A. N. Schechter. Academic Press, New York, 1972. pp. x+255. £3.

Transport and Accumulation in Biological Systems. 3rd ed. By E. J. Harris. Butterworths, London, 1972. pp. vii+454. £8.50.

Chromosome Techniques. Theory and Practice. 2nd ed. By A. K. Sharma and A. Sharma. Butterworths, London, 1972. pp. 575. £12.

Fd Cosmet. Toxicol. Vol. 11, pp. 503–509. Pergamon Press 1973. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

TOO MANY FOODS IN PURSUIT OF TOO FEW PRESERVATIVES

So, naturalists observe, a flea
Has smaller fleas that on him prey;
And these have smaller still to bite 'em,
And so proceed *ad infinitum*

Jonathan Swift (1667–1745)

From time immemorial, man has been required to respond to the challenge of a fluctuating food supply and, in devising methods for food storage, to develop techniques designed ultimately to diminish the risk of microbial and autolytic spoilage. It is freely recognized that increasing urbanization of populations in so-called developed countries produces its own particular difficulties with regard to food supply. Roughly speaking, three-quarters of the UK population is urban and over one-third of that population is accommodated in towns or conurbations in excess of 100,000 inhabitants. Clearly, it is impractical to feed such populations entirely on fresh food, especially since a very substantial part is in fact imported. The net consequence is that urban areas ultimately face a situation closely akin, in practical terms, to the problem of the redistribution of the world's food wealth.

The technology of food preservation has become increasingly refined in bulk industrial terms and yet, in principle, differs little from an art which has emerged through necessity, and in many cases by chance, over the last several thousand years. This is certainly true of physical methods of preservation, with the notable exception of irradiation. Equally, and paradoxically for a society with a highly developed chemical technology, the art of chemical preservation has seen few major fundamental advances. Time-honoured methods of a cottage-based food economy involving, for example, significant changes in the pH of the food matrix ("pickling") or elevation of electrolyte or other solute concentration with consequent osmotic changes ("salting") are still much in vogue. The same is also true of the use of fermentation processes designed to produce a preferred chemical state *in situ*. Moreover, few food technologists would be so unwise as to pinpoint a major innovation in the principles of use of specific, reactive chemical agents for the sterilization and preservation of food products.

In an editorial written exactly 7 years ago (*Cited in F.C.T.* 1966, **4,** 187), attention was drawn to the fact that a predictable consequence of the improvement in dietary standards of an affluent, urban society was an increased intake of specific preservatives. This view has been echoed in the recently published Report on the Review of the Preservatives in Food

Regulations 1962 (*ibid* 1973, **11,** 117). In redefining the term "preservative", high-lighting at the same time how restricted is the range of chemicals approved for this purpose, the Report throws into sharp relief the areas of concern relating to the use of nitrate and nitrite and the problem of nitrosamines. Inevitably, attention has also been focused on the use of sulphur dioxide (SO_2) and sulphites (SO_3^{2-}) as preservatives of widespread application, as well as inhibitors of non-enzymic browning of foodstuffs. While prepared to "advise exceptionally" that SO_2 and SO_3^{2-} should remain classified as "additives that the available evidence suggests are acceptable for use in food", the Report also cautions that "the calculated average daily intake of sulphur dioxide per person is . . . very near the estimated acceptable daily intake".

With relatively few exceptions, recent toxicological and basic biological studies on SO_2 and SO_3^{2-} have fallen into two major categories—multigeneration studies and other investigations of the long-term effects in animals of SO_2 and SO_3^{2-} *per se*, and observations on the potential toxic hazard that might result from the interaction of SO_2 and SO_3^{2-} with macromolecules of intrinsic biological importance.

In the first category, the studies by Til *et al.* (*Fd Cosmet. Toxicol.* 1972, **10,** 291 & 463) in the rat and pig confirm the view that SO_3^{2-} added freshly to diets is relatively innocuous to experimental animals maintained under closely controlled laboratory conditions. Equally, substantial advances have been made in the study of the reactivity of SO_2 and SO_3^{2-} with vitamin components in foods. Thus, the inactivation of thiamine in the diet, which was held to be largely responsible for the effects of SO_2, has been intensively studied by Cremer and his group (see, for example, Hötzel *et al. Int. Z. VitamForsch.* 1969, **39,** 372 and Bitsch *et al. ibid* 1969, **39,** 384). Clear evidence has now been advanced showing that animals on nutritionally adequate diets can withstand, with regard to thiamine destruction, substantial intakes of SO_3^{2-}. The same group (Muskat & Bitsch, *ibid* 1970, **40,** 46; Cremer & Hötzel, *ibid* 1970, **40,** 52) has also confirmed the results of a BIBRA study on human subjects, which established that oral administration of dose levels equivalent to 200 mg SO_2/day in lime juice did not produce symptoms of thiamine deficiency or any change in the urinary excretion of thiamine. On the other hand, the reported interaction between SO_3^{2-} and folic acid (Vonderschmitt *et al. Archs Biochem. Biophys.* 1967, **122,** 488) remains to be assessed in nutritional terms.

In the second category, and at the other extreme, several groups have examined the interaction of SO_3^{2-} with nucleic acids *in vitro* and have raised the spectres of possible interference with double-helix formation and inactivation of messenger RNA in coding for protein synthesis (Shapiro & Braverman, *Biochem. biophys. Res. Commun.* 1972, **47,** 544), as well as of mutagenic effects on micro-organisms (Inoue *et al. Chemico-Biol. Interactions* 1972, **5,** 85). In addition, it has been shown that the administration of gaseous SO_2, albeit at relatively high concentration, to human lymphocytes in culture reduced cell size, DNA synthesis and mitotic indices and produced certain chromosome abnormalities (Schneider & Calkins, *Envir. Res.* 1970, **3,** 473). Of some significance in relation to these findings is the report that gaseous SO_2 administered to experimental animals is scavenged efficiently by the formation of *S*-sulphoprotines in the blood stream, presumably by the sulphitolysis of disulphide bridges (Gunnison & Benton, *Archs envir. Hlth* 1971, **22,** 381). However, neither the biochemical consequence for proteins whose structural integrity has been disturbed in this way nor, indeed, the subsequent metabolic fate of the structures modified *in vivo* has been followed.

These areas are obviously worthy of further investigation since, in the light of past

experience, each is potentially a focus for academic and public concern. However, the immediate relevance of such work to SO_2 and SO_3^{2-} consumed in the diet is open to question. There is, for example, no indication of the extent to which SO_2 and SO_3^{2-} exist unaltered after food treatment. Thus, apart from the autoxidation of these reactive species to sulphate, there are many individual food constituents which have the capacity to interact with them to produce derivatives of varying chemical stability. Notable candidates for such reactions include aldehydes, ketones, unsaturated alkyl residues and thiol moieties (Schroeter in *Sulfur Dioxide, Applications in Foods, Beverages and Pharmaceuticals*, p. 105, Pergamon Press, Oxford, 1966). Unfortunately, rather than simplifying the situation, these products constitute a series of toxicological imponderables in any assessment of the safety-in-use of the original preservatives. In this context, the results of preliminary research at BIBRA strongly indicate that the presence of carbohydrate hydroxysulphonates in SO_3^{2-}-treated diets subjected to prolonged storage may contribute materially to the acute phenomena, including diarrhoea and stunting of growth in weanling animals, first observed by Bhagat & Lockett (*Fd Cosmet. Toxicol.* 1964, **2,** 1). Equally, it would be at best unwise to ignore the possibility that the long-term administration of stable sulphonates derived originally from unsaturated long-chain alkyl compounds or *S*-sulphoaminoacids in peptides and proteins might have as yet unsuspected toxicological and nutritional consequences. Manifestly, the factors which influence the formation of such materials in stored food, their stability *in vivo* and the pharmacodynamics of their absorption, metabolism and excretion in animal species and man are all topics that require the expenditure of further research effort.

Finally, there is a body of evidence that mammalian tissues have ample capacity to oxidize SO_3^{2-} to sulphate, and some biochemical characterization of the enzyme system involved, known trivially as sulphite oxidase, has been achieved (Cohen & Fridovich, *J. biol. Chem.* 1971, **246,** 359 & 367; Cohen *et al. ibid* 1971, **246,** 374). Regrettably, the system is not immutable and observations on a child apparently congenitally deficient in liver sulphite oxidase activity have been reported (Irreverre *et al. Biochem. Med.* 1967, **1,** 187). In common with many other inborn metabolic errors, the resultant pathological effects included neurological abnormalities, leading in this case to virtual decortication and subsequent death. It is particularly noteworthy that three of the patient's seven siblings died in infancy with pathological changes of the central nervous system but, unfortunately, before biochemical examination could be made. Those who have learned the lessons taught by the study of other inherited metabolic defects will appreciate that such findings provide adequate warning that sulphite oxidase activity is likely to vary substantially even in otherwise normal humans. Moreover, there is evidence that in the rat, at least, the embryo and neonate are virtually devoid of the enzyme, full activity appearing only some time after birth (Joshi *et al. Biochem. J.* 1969, **111,** 297). The behaviour of the system under load as a function of age, its inducibility or repressibility, the effects of pregnancy, and factors that might influence its foetal or postnatal development in man are notable as other areas of ignorance.

There can be little doubt that there are several gaps in our knowledge. These must be filled, especially since hard on the heels of nitrite, ethylene oxide and diethyl pyrocarbonate, each with its own particularly vexing problems, come SO_2 and SO_3^{2-} equally determined to display a more diverse range of biological activity. At the end of the day one is left to wonder how many will be stimulated to reiterate the words of a contemporary toxicologist:

"Heaven preserve us from preservatives!"

CUTANEOUS REACTIONS TO CHROMIUM

Chromium (Cr) is one of the commonest causes of skin allergy, playing a causative role in some 8–9% of all cases seen in dermatological clinics (*Cited in F.C.T.* 1969, **7,** 271; *ibid* 1973, **11,** 147). The proportion so afflicted is even greater when women are excluded from the count, reaching 12% in Scandinavian male patients (Magnusson *et al. Acta derm. vener., Stockh.* 1968, **48,** 110) and 11% in European men generally (Fregert *et al. Trans. a. Rep. St John's Hosp. derm. Soc., Lond.* 1969, **55,** 17). The levels in the latter study varied from city to city and attained a record 21% in Bari, in southern Italy, where a large number of dermatological patients came from the building and construction works in the area.

The presence of Cr in cement is responsible for the high incidence of allergy in the building industry, while its use in primer paints and anti-rust coatings and in alloys and electrodes is the bane of car and foundry workers respectively (*Cited in F.C.T.* 1964, **2,** 219 & 396; *ibid* 1969, **7,** 91). Another hazardous place of employment from the point of view of Cr sensitization is a sulphite-pulp factory, where the chemicals used—sodium sulphate and calcium oxide and carbonate—may be highly contaminated with Cr in both valency states (Fregert *et al. Acta derm.-vener., Stockh.* 1972, **52,** 221). Two men engaged for 1 and 15 years, respectively, in the recovery process at such a factory developed eczema of the face and hands, which patch-testing showed to be due to Cr. Analysis showed levels of Cr^{VI} as high as 3300 μg/g in "chrome-cake", a form of sodium sulphate obtained as a by-product of chromic acid processing, and other technical- and even analytical-grade samples of this chemical were found to contain Cr^{VI} and/or Cr^{III}, although at much lower levels.

The Cr in steel is rarely the cause of allergy, but a building worker already sensitized to Cr suffered a severe recurrence of his dermatitis when he transferred to the steel store of an engineering plant (Weiler, *Berufsdermatosen* 1969, **17,** 316). There he had contact with surface-corroded steel containing 1·6% Cr, which could readily be extracted by the mineral oil used as a lubricant. Further work revealed that Cr was frequently present in used mineral oils, either as a powder or in solution (Rüssel & Weiler, *ibid* 1971, **19,** 23). Cr was also detected in quicklimes and in glues and gelatins, which had presumably become contaminated during the calcination process (Weiler & Rüssel, *ibid* 1971, **19,** 292). Six quicklimes analysed contained 2·4–46 ppm Cr, of which 18–42% was Cr^{VI}, while in seven glues and gelatins derived from hide and bone the Cr content varied from 0·08 to 0·68 ppm. One further sample of glue of unknown origin (probably from leather waste) contained as much as 17 ppm Cr. In view of the widespread application of these products, the potential for sensitization is considerable.

Another more bizarre source of Cr dermatitis, this time non-occupational, is the pigment used for tattooing. Adverse reactions to light-blue tattoos have been largely associated with cobalt pigments (*Cited in F.C.T.* 1970, **8,** 118), while in the case of green tattoos Cr may be the guilty party. Tazelaar (*Dermatologica* 1970, **141,** 282) reports on the case of a 58-year-old man who had been gaily tattooed some 15 years previously in red and blue. For 10 years he had suffered from a dry eczema of the palms of the hands, and for the last few years there had been some itching and firm nodules in the light-blue parts of the tattoo. Large amounts of Cr were demonstrated spectroscopically in these areas, but cobalt could not be detected, although patch tests with 0·5% potassium dichromate ($K_2Cr_2O_7$) and 2% cobalt chloride were both strongly positive. Unfortunately, identification of the precise Cr compound involved was not possible.

Even housewives are not immune from the pervasive effects of Cr, since it is present in

detergents. A survey of hand dermatitis among the housewives of five northern European cities indicated that Cr was involved in 5% of such cases (*Cited in F.C.T.* 1971, **9,** 760). In contrast, Feuerman (*Acta derm.-vener., Stockh.* 1969, **49,** 288) found that as many as 47 of 50 similarly-afflicted Israeli housewives responded positively to Cr in patch-tests. This was so surprising that the investigation was extended to 250 such housewives, of whom 229 (91·6%) reacted positively to Cr (*idem, Dermatologica* 1971, **143,** 292). Analysis of 14 detergents widely used in Israel revealed a Cr content in the range 0·4–23·2 μg/g, levels which did not appear to be higher than in other countries. The affliction was confined to exposed areas, and it was concluded that the most likely explanation for the high incidence of Cr dermatitis in Israel was the sunny climate. There has been little indication in the past that Cr dermatitis may be exacerbated by light, but Tronnier (*Arch. klin. exp. Derm.* 1970, **237,** 494) found that sufferers from Cr dermatitis showed an erythematous response to ultraviolet radiation at a lower threshold than did healthy individuals (although an increased sensitivity was also manifested by persons suffering from other skin diseases). When a 0·2% solution of Cr^{VI} (as $K_2Cr_2O_7$) or Cr^{III} (as $KCr(SO_4)_2$) was applied before irradiation to the skin of 25 Cr-dermatitis sufferers and 20 normal individuals, Cr^{III} resulted in an immediate lowering of the erythematous threshold, while Cr^{VI} appeared to have an initial light-protective effect which was reversed after 24 hours. Tronnier (*loc. cit.*) suggests that irradiation may bring about the oxidation of Cr^{III} to Cr^{VI}, which penetrates the skin more readily. It may then be reconverted to Cr^{III} within the skin, where an antigenic complex can be formed (*Cited in F.C.T.* 1964, **2,** 398).

Despite this conventional version of the mechanism whereby Cr^{VI} is in practice a much more potent sensitizer than Cr^{III}, Bang Pedersen *et al.* (*Acta derm.-vener., Stockh.* 1970, **50,** 431) found no measurable disappearance of 20·8 μg Cr^{VI} applied as an aqueous solution of $Na_2{}^{51}CrO_4$ for 48 hours under a test patch to the forearms of two Cr-sensitive and two non-allergic subjects. Nor did the blood cells or plasma show any evidence of Cr absorption. Most of the applied Cr evidently remained in the patch, since its removal left only 7·8–10·3% of the initial quantity on the skin, and it is possible that the cellulose it contained may have brought about the reduction of Cr^{VI} to Cr^{III}. After a month, the residual amounts of Cr persisting in the skin of the non-allergic subjects were several times greater than the amounts persisting in the allergic subjects, probably because, in the latter, more of the epithelium and applied Cr had been shed as a result of the allergic reaction.

Absorption of small amounts of both Cr^{VI} and Cr^{III} through excised human mammary skin was demonstrated *in vitro* by Wahlberg (*Dermatologica* 1970, **141,** 288) using $^{51}CrCl_3$ and $Na^{51}CrO_4$ at concentrations of 0·017–0·398 M. However, there appeared to be no difference in absorption between the two valency forms. Moreover, the previous finding that at concentrations above 0·261 M significantly more Cr^{VI} than Cr^{III} was absorbed through guinea-pig skin *in vivo* (*Cited in F.C.T.* 1966, **4,** 541) could not be confirmed *in vitro*. The absolute absorption of both Cr^{III} and Cr^{VI} increased with increasing concentration, as shown by measurements of disappearance over 24 hours. Comparison with earlier results (*ibid* 1966, **4,** 541) indicated that Cr^{III} was absorbed through guinea-pig skin more readily *in vitro* than *in vivo*, as was Cr^{VI} at low concentrations, but at high concentrations Cr^{VI} showed a greater absorption *in vivo*. The pH changes of the unbuffered solutions containing Cr^{VI} were greater *in vitro* than *in vivo*, and it is possible that Cr^{VI} may have been reduced more readily *in vitro* to Cr^{III}.

Schwarz-Speck & Grundmann (*Arch. derm. Forsch.* 1972, **242,** 273) found that when guinea-pig skin was incubated in a $K_2Cr_2O_7$ solution the decline in Cr^{VI} concentration was

strongly dependent on pH, a greater decrease being observed in acid solution (pH 5·4) than under neutral or alkaline conditions. The buffer system used also had some influence. The skin of sensitized guinea-pigs produced no greater reduction of Cr^{VI} than did that of control animals, although highly sensitized skin was apparently somewhat more effective than that of non-reactive guinea-pigs. The difference was, however, relatively slight.

The simultaneous use of 2% Triton X-100 (TX) increases the allergic response to externally applied Cr^{III} in guinea-pigs, presumably because it enhances percutaneous absorption (*Cited in F.C.T.* 1966, **4,** 230). Schwarz-Speck & Grundmann (*loc. cit.*) report that a very high proportion of guinea-pigs could be sensitized by ten epicutaneous applications of Cr^{III} (as 5% $Cr_2(SO_4)_3$) in 2% TX, positive reactions being obtained in subsequent patch tests to 0·5% Cr^{III} in TX in 27 of 29 animals and to 0·5% Cr^{VI} in all 29. Fewer guinea-pigs were sensitized by an initial five applications of Cr^{VI} (as 5% $K_2Cr_2O_7$) to defatted skin, 23 of 27 later reacting positively to 0·5% Cr^{III} in TX and 20 of 27 to 0·5% Cr^{VI}. In both of these experiments the patch-test responses to Cr^{III} and Cr^{VI} were qualitatively and quantitatively very similar.

Despite the efficacy of TX on guinea-pig skin, Valér & Rácz (*Berufsdermatosen* 1971, **19,** 302) found no increase in allergic response to Cr^{III} in 60 dermatological patients, including 20 responding positively to patch tests with Cr^{VI}, when other penetration-enhancing methods were used. These involved applying the Cr^{III} in aqueous solutions of 1–10% sodium lauryl sulphate or 25–100% dimethyl sulphoxide (DMSO), or stripping the skin with adhesive tape. A large number of positive reactions to Cr^{III} in 75 or 100% DMSO were in all cases attributed to the irritancy of these high concentrations of the vehicle, as were the smaller number of reactions seen with the other methods. Nor did increasing the concentration of $CrCl_3$ in the range 0·074–1·0 M lead to a significant increase in response. Tests with 0·5–1·0 M-$CrCl_3$ on intact skin were positive in only four cases, in three of which there had been occupational exposure and resulting sensitization to Cr^{VI}. The fourth case, a tanner, did not react to Cr^{VI} in patch tests and had been occupationally exposed only to Cr^{III}, but he showed no response to intracutaneously administered Cr^{III}. Patch tests on a further 60 patients indicated that the positive reactions seen in these four cases were in all probability due to irritation rather than sensitization, since of 46 subjects not occupationally exposed to Cr, and giving no response to patch tests with $K_2Cr_2O_7$, three reacted to 0·5 M-$CrCl_3$ and nine to 1·0 M-$CrCl_3$. However, the possibility of some degree of cross-sensitization between Cr^{VI} and Cr^{III} could not be entirely ruled out.

A further study of 249 dermatological patients, including 199 occupationally exposed to Cr, failed to confirm that Cr^{III} rather than Cr^{VI} is the primary allergen (*idem, ibid* 1972, **20,** 24). Of the 199, 164 reacted to intracutaneously injected 0·001 M-$K_2Cr_2O_7$, and 37 (a mere 22·56% of those reacting to Cr^{VI}) to 0·01 M-$CrCl_3$ administered in the same fashion. Only four of the 37 gave stronger reactions to Cr^{III} than to Cr^{VI}, and two of these four also responded to epidermally applied 0·5 M-$CrCl_3$. Since 0·1 M-$CrCl_3$ produced necrosis when given intracutaneously, it was concluded that the reactions to 0·01 M-$CrCl_3$ were probably indicative of irritation rather than allergy and may have reflected the altered reactivity of the skin of Cr^{VI}-sensitized patients to other metallic salts; alternatively they could have been "pseudo-specific" reactions joining the reactions of the more potent sensitizer Cr^{VI}. In either case the far greater response to Cr^{VI} would suggest that no transformation to Cr^{III} need take place before an allergic reaction can occur.

The reliability of the experimental methods used is a great problem in all studies of this type. Before embarking on a study of transferred allergy in guinea-pigs, Skog & Wahlberg

(*Acta derm.-vener., Stockh.* 1970, **50,** 103) wisely conducted an evaluation of the methods used to induce Cr allergy in this species and to detect allergy once induced. Of three alternative methods of sensitization the most effective proved to be that devised by Polák & Turk (*Clin. exp. Immunol.* 1968, **3,** 245) in which five simultaneous im injections of 0·1% $K_2Cr_2O_7$ at different sites (total 1 mg $K_2Cr_2O_7$) are followed 2 weeks later by a single intracutaneous injection of 0·025% $K_2Cr_2O_7$, a procedure repeated four times at weekly intervals and accompanied by skin painting with 0·5% $K_2Cr_2O_7$ in TX. Detection methods in which $K_2Cr_2O_7$ was injected intracutaneously or applied to the skin in a petrolatum ointment gave more reliable results than did patch tests, and painting and 'nipple tests' were rejected as unsatisfactory. The best of these methods were then used to assess the passive transfer of Cr allergy and dinitrochlorobenzene (DNCB) allergy in guinea-pigs by cutaneous parabiosis and by arteriovenous cross transfusion (Skog & Wahlberg, *Acta derm.-vener., Stockh.* 1970, **50,** 189). In the parabiosis experiments, six of the seven recipients were successfully sensitized to Cr and seven of 15 to DNCB, while cross transfusion was successful in four out of ten cases of Cr allergy and six out of nine cases of DNCB allergy. The microscopic findings were in all cases used as criteria, although they did not always correspond with the macroscopic evidence, and discrepancies were also evident between intracutaneously and epicutaneously elicited reactions.

Allergic reactions to Cr are normally confined to the skin, but there have been sporadic reports of asthma associated with industrial exposure. Williams (*N. Carol. med. J.* 1969, **30,** 482) describes two additional cases, one due to exposure to chromic acid fumes in a textile-engraving firm and the other to zinc chromate primer paint spray in a machinery factory. In the first case there were also eczematous skin lesions, which appeared some months before the pulmonary symptoms.

An interesting study by Samitz (*Acta derm.-vener., Stockh.* 1970, **50,** 59) suggests that the dermal and other toxic effects of Cr^{VI} could be prevented or substantially alleviated by the use of ascorbic acid (AA). Acute oral Cr^{VI} intoxication in rats was averted by AA given within 2 hours of an otherwise effective dose of $K_2Cr_2O_7$, and the skin ulcers produced by a strong solution of Cr^{VI} in guinea-pigs healed significantly more rapidly when promptly washed with 10% aqueous AA (although distilled water had little effect). A regime involving the soaking of hands and forearms in 10% AA after the handling of Cr^{VI} mixtures was also effective in protecting sensitized workers in the printing and lithographing industries, and in three of five cases led to a complete disappearance of symptoms. The efficacy of respirator filters against chromate mist was enhanced by up to 50% when they were impregnated with 20% AA; 10% AA was almost as effective, but lost its activity more rapidly. *In vitro* tests showed that Cr^{VI} was inactivated by AA through reduction to Cr^{III} and subsequent complex formation. A study such as this may be of practical significance, and perhaps deserves some consideration by those industries in which Cr exposure presents a problem.

Fd Cosmet. Toxicol. Vol. 11, pp. 511–523. Pergamon Press 1973. Printed in Great Britain

TOXICOLOGY: ABSTRACTS AND COMMENTS

EMULSIFIERS AND STABILIZERS

2522. Brominated oils and the heart

Munro, I. C., Hand, B., Middleton, E. J., Heggtveit, H. A. & Grice, H. C. (1972). Toxic effects of brominated vegetable oils in rats. *Toxic. appl. Pharmac.* **22,** 432.

Short-term toxicity studies on brominated cottonseed oil (BCSO) and the biochemical and pathological changes found in rats fed low dietary levels of BCSO have already been reported (*Cited in F.C.T.* 1972, **10,** 258; Munro *et al. Fd Cosmet. Toxicol.* 1971, **9,** 631). Short-term toxicity studies in rats have been extended by the same Canadian group to include other brominated vegetable oils, namely brominated corn (BCO), olive (BOO) and sesame (BSO) oils.

Groups of 15 male and 15 female rats were maintained under normal laboratory conditions and fed diets containing 0, 0·1 or 0·5% of one of the four brominated oils named above for 105 days. Food consumption was significantly increased in female rats receiving 0·5% BSO in the diet, but otherwise food consumption and weight gain were unaffected in all groups. Relative liver and kidney weights were significantly increased ($P<0·01$) in all rats receiving 0·5% brominated oils. The relative heart weights were significantly increased ($P<0·01$) in all rats fed 0·5% brominated oils with the exception of male rats receiving 0·5% BOO. The changes in organ weights were generally associated with pathological changes in these organs. Cardiac lesions, characterized by degenerating myocardial cells containing small fat vacuoles in the cytoplasm and in some cases myocytolysis, were observed in all rats given 0·5% brominated oils but were most apparent in rats fed BSO, while 0·5% BOO appeared to be less cardiotoxic than the other oils to male rats. Some minimal changes were also seen among the rats given the 0·1% diets, particularly in those fed 0·1% BSO. Renal changes were confined to male rats fed 0·5% brominated oils and consisted of mild vacuolization of the proximal tubular epithelium. With the exception of BOO, the brominated oils produced marked rises in histologically detectable levels of liver lipids, particularly in the groups fed the 0·5% dietary levels. Microfollicular hyperplasia of the thyroid was frequently observed in treated animals, but was more prevalent in females than in males.

The authors suggest that the fatty change and subsequent myocytolysis associated with feeding high doses of brominated oils may be due, in part, to a relative intracellular hypoxia, but further studies will be required to elucidate the exact mechanism responsible for the reported effects.

ANTIOXIDANTS

2523. Favourable reports on Ionox compounds

Wright, A. S. & Crowne, R. S. (1972). The alimentary absorption of 2,4,6-tri(3,5-di-*tert.*-butyl-4-hydroxybenzyl)phenol (Ionox 312) in the rat. *Xenobiotica* **2,** 1.

Wright, A. S., Crowne, R. S. & Potter, D. (1972). The fate of 1,3,5-tri(3,5-di-*tert.*-butyl-4-hydroxybenzyl)-2,4,6-trimethylbenzene (Ionox 330) in rats fed the compound over a prolonged period. *Xenobiotica* **2,** 7.

Compounds belonging to the Ionox series of hindered phenolics have been found to act as general-purpose antioxidants which essentially lack toxicity when administered to animals by the oral or percutaneous route. Previous reports have shown that Ionox 330 is quantitatively eliminated in the faeces within 48 hr in dog and man and in 72 hr in the rat, and undergoes no material absorption or metabolism (*Cited in F.C.T.* 1966, **4,** 228).

In the first investigation cited above, rats were given a 6·8 mg dose of Ionox 312 labelled with carbon-14, by means of a stomach tube. No radioactivity appeared in the exhaled air, and the urine contained no more than 0·04% of the total radioactivity administered. A 5 g sample of omental fat from a female killed 6 hr after dosing contained 0·03% of the total activity, but other fat samples contained none. After removal of the alimentary tract, only small amounts of ^{14}C activity remained in the carcass, and only traces appeared in bile. The findings suggest that Ionox 312 may undergo slow oxidation within the animal gut, the oxidation products being absorbed to a limited extent.

The second paper cited is concerned with rats fed from weaning on a diet containing 0·2% Ionox 330 for up to 200 days, and with the fate of a single dose of 6·8 mg of the same antioxidant labelled with ^{14}C and given by stomach tube to these Ionox-fed rats. No radioactivity appeared in expired air, and only traces in the urine (up to 0·02% in 48 hr). Very small quantities of activity were detected in the body fat and liver. Almost all the single test dose was recovered unchanged in the faeces. A small proportion appeared to be oxidized in the gut to its triquinone methide, and the oxidation products were absorbed to a limited extent. This oxidation and absorption were independent of dose level, since similar results were obtained in rats pretreated with dietary Ionox 330 and then given a labelled dose equivalent to 20 or 400 mg/kg body weight. The oxidation of Ionox 330 seemed unlikely to involve any enzymatic mechanism, at least in its early stages.

[In summary, these studies support previous evidence that the Ionox antioxidants are virtually inert when consumed, and they provide no evidence of any induction of enzyme synthesis within the gut.]

AGRICULTURAL CHEMICALS

2524. 2,4,5-T, 2,4-D and the foetus

Khera, K. S. & McKinley, W. P. (1972) Pre- and postnatal studies on 2,4,5-trichlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and their derivatives in rats. *Toxic. appl. Pharmac.* **22,** 14.

2,4-Dichlorophenoxyacetic acid (2,4-D) has been shown to possess a low toxic potential when fed for a prolonged period to rats and dogs (*Cited in F.C.T* 1972, **10,** 721) and both 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) seem to be well tolerated by the chick (*ibid* 1973, **11,** 149). The study cited above concentrated on the possible toxicity of these herbicides to the foetus and newborn animal, an aspect which has been the subject of conflicting reports, at least as far as 2,4,5-T is concerned (*ibid* 1972, **10,** 722).

2,4-D and 2,4,5-T were given to rats in daily oral doses suspended in aqueous gelatin or

corn oil during days 6–15 of gestation. The samples of 2,4,5-T tested contained less than 0·5 ppm of the known teratogenic contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Two samples of 2,4,5-T had adverse effects on the weight gain of the pregnant rats when given at the 150 mg/kg level, and one of these samples killed three of the eight treated dams. Three 2,4,5-T samples (including these first two) had no detectable toxic effects in doses of 100 mg/kg. Four samples of 2,4-D, and samples of its butyl and isooctyl esters and butoxyethanol and dimethylamine salts, did not affect maternal weight gain at dose levels up to 150 mg/kg (or 300 mg/kg in the case of the dimethylamine salt).

At the 100 and 150 mg/kg levels, 2,4,5-T significantly reduced foetal weight and increased the incidence of foetal death and skeletal abnormalities. Little or no effect occurred with doses of 25 and 50 mg/kg. The butyl ester of 2,4,5-T had no evident effect at either dose level (50 and 150 mg/kg) tested. At the 100 and 150 mg/kg levels, 2,4-D produced foetal damage similar to that caused by 2,4,5-T. At the highest dose levels all the derivatives of 2,4-D increased the incidence of terata, and the butyl and isooctyl esters tended to depress foetal weight. There was no evidence that 2,4,5-T in doses up to 100 mg/kg affected the sex ratio, viability or weight gain of the newborn litters. 2,4-D within the same dose range did not affect survival or have any consistent influence on body weight.

Overall, the findings in the pre- and postnatal studies indicate that the postnatal effects of 2,4,5-T and 2,4-D are not related to the teratogenic potential of the two compounds.

2525. Pesticides in pregnant pigs

Uzoukwu, M. & Sleight, S. D. (1972). Effects of dieldrin in pregnant sows. *J. Am. vet. med. Ass.* **160,** 1641.

Although organochlorine pesticides are widely used for agricultural purposes, their effect on certain farm animals has not been examined as thoroughly as might be desirable. We have previously referred to demonstrations of the placental transfer of these compounds in pregnant mice (*Cited in F.C.T.* 1965, **3,** 656) and rabbits (*ibid* 1969, **7,** 536), and the present authors have examined the effects in pregnant sows given oral doses of dieldrin.

Repeated oral doses of up to 15 mg dieldrin/kg were administered to the animals during the last 30 days of gestation, and in the case of two of the sows, sodium nitrite was also administered sc at a rate of 25 mg/kg. Postmortem examination of the sows, which were sacrificed at the end of the experimental period, revealed that although dieldrin concentrations in foetal liver and brain were significantly lower than those in maternal tissues, placental transfer had obviously occurred. Mild degeneration of the renal tubules and slight hepatic lipidosis were observed in the sows but no lesions were detected in the foetuses. No foetal deaths or abortions occurred in sows given dieldrin alone, or in those also given nitrite.

2526. Blowfly larva control unsuitable for hens

Sherman, M., Beck, J. & Herrick, R. B. (1972). Chronic toxicity and residues from feeding Nemacide [*O*-(2,4-dichlorophenyl)*O*,*O*-diethyl phosphorothioate] to laying hens. *J. agric. Fd Chem.* **20,** 617.

Certain insecticides that are useful in controlling the development of fly larvae in poultry droppings have been found in short-term toxicity tests to be relatively harmless to the birds themselves. The present paper examines the extent to which one of these pesticides accumu-

lated in the tissues of laying hens, in order to investigate any potential hazard associated with its more prolonged use.

Technical-grade Nemacide (*O*-(2,4-dichlorophenyl)-*O*,*O*-diethyl phosphorothioate) was incorporated into the diet of laying hens for 55 wk, at levels ranging from 50 to 800 ppm. Efficient control of housefly and blowfly larvae was achieved with 50 ppm Nemacide, though higher levels were required for the control of other species. Although 23% of the hens died during the experiment, there was no direct correlation between mortality and the level of pesticide used. There was, however, a direct relationship between Nemacide concentration and the degree of inhibition of cholinesterase activity in the plasma. No significant effects were observed on body weight or feed consumption, or on the production, weight or quality of the eggs, but ingestion of a dietary level of 800 ppm Nemacide imparted an undesirable flavour to the eggs.

Gas chromatography, using an electron-capture detection method, was used to measure nanogram quantities of Nemacide and its metabolite, 2,4-dichlorophenol. Significant quantities of Nemacide were found in the liver, muscle, fat and eggs of treated hens, but the metabolite was detected only in the liver and egg yolk.

The authors consider that although a level of 50 ppm Nemacide may be effective against fly larvae and has no detrimental effect on the birds or their eggs, the residues and metabolite found in some tissues makes the insecticide unsuitable for use in the feed of laying hens.

THE CHEMICAL ENVIRONMENT

2527. Adverse effects of nickel sulphate in male rats

Waltschewa, W., Slatewa, M. u. Michailow, I. (1972). Hodenveränderungen bei weissen Ratten durch chronische Verabreichung von Nickelsulfat. *Exp. Pathol.* **6,** 116.

We noted earlier how improvements in workshop practice have successfully overcome the hazards of lung and nasal cancer, which were once closely associated with employment in a South Wales nickel (Ni) refinery (*Cited in F.C.T.* 1972, **10,** 113). However, published observations of degenerative, although reversible, changes produced in the testes of experimental animals by single injections of nickel sulphate ($NiSO_4$) prompted the authors named above to carry out a long-term study in order to provide further information on possible hazards to male workers employed in an environment in which they are persistently exposed to Ni compounds.

Male white rats were given 25 mg $NiSO_4$/kg daily by stomach tube for 120 days. At the end of this period they were caged with females in oestrus and then killed for histological and histochemical examination of the liver, kidneys and testes. Only slight changes in the activity of certain liver enzymes and similarly slight degenerative changes in the cells of the liver and renal tubules were seen in the treated rats, but the testes were smaller than those of the controls and showed severe lesions of the parenchyma. Microscopic examination of the testes showed a proliferation of connective tissue in the interstitial region and an increase in the number of interstitial cells, accompanied by a reduction in spermatogenesis. Motility of the spermatozoa was also reduced. In line with these findings was the fact that although the control group fertilized three out of six females, the treated animals produced no offspring. Studies on homogenates of the rat testes indicated that $NiSO_4$ treatment had affected

the activity of several enzymes, and disturbed the pattern of lactic-dehydrogenase isoenzyme distribution.

2528. Aromatic metabolism in the gut

Scheline, R. R. (1972). The metabolism of some aromatic aldehydes and alcohols by the rat intestinal microflora. *Xenobiotica* **2,** 227.

It is known that the major metabolic route for many aromatic aldehydes and primary alcohols involves oxidation to the corresponding acid. Reduction to the alcohol is likely to occur much less readily. Since the micro-organisms present in the gut are capable of numerous reductive reactions, the present work was designed to investigate their role, if any, in the metabolism of selected benzene derivatives in rodents.

Various benzaldehyde and benzyl alcohol derivatives, containing hydroxy and methoxy groups, were incubated with caecal extracts from the rat. Reduction of the aldehydes to the alcohol and oxidation to the corresponding acid constituted the normal pattern of metabolism, with the reductive pathway predominating. Either pre-warming the cultures or pre-incubating the caecal extracts with various antibiotics appeared to inhibit the oxidative reaction to a much greater degree than the reductive route.

Many of the aromatic alcohols showed little metabolic change, but a very limited degree of oxidation to benzoic acid generally occurred and where a compound contained a *p*-hydroxy group, reduction to toluene derivatives always occurred. *O*-Demethylation occurred with disubstituted benzyl alcohols. Polar metabolites that formed from several aldehydes, though not from benzoic acid derivatives or benzyl alcohol derivatives, were postulated to be 1,1-diols (aldehyde hydrates), since they easily reverted to the original aldehyde.

The *in vivo* role of the intestinal flora in the metabolic transformation of these aldehydes and alcohols was concluded to be a minor one compared with that of the tissue enzymes because of the relative importance of oxidation *in vivo*, but several additional metabolites may be formed in small quantities in the gut. The relative contribution of the intestinal reactions to the overall metabolism of these compounds when they are administered to animals is currently being evaluated.

2529. Absorbing the aliphatic carbamates

Houston, J. B., Upshall, D. G. & Bridges, J. W. (1972). The absorption of aliphatic carbamates by everted gut sacs of rat. *Biochem. J.* **128,** 141P.

Carbamates of the general structure $RO{\cdot}CO{\cdot}NH_2$ in which R is an aliphatic group ranging from methyl to *n*-heptyl, show a wide range of lipophilicity and do not undergo ionization or hydrolysis in aqueous solution. The best known member of this group is perhaps the ethyl compound, commonly known as urethane. Carbamates in concentrations of 10–50 mM were incubated in oxygenated Krebs-Henseleit medium at 37°C in cannulated everted sacs of rat gut and were sampled by gas–liquid chromatography every 10 min for 1 hr.

Recoveries averaging 97% indicated a lack of metabolism. The percentages of the initial carbamates in the mucosal fluid that were absorbed/hr/cm^2 were: methyl, 2·81; ethyl, 3·37; *n*-propyl, 5·07; *n*-butyl, 6·60; *n*-pentyl, 3·75; *n*-hexyl, 2·25; *n*-heptyl, 0·67. The absorption rate of the highly water-soluble compounds was not affected by the 5% Tween 80 in the medium, and all carbamates remained in solution throughout the experiment. Estimation of the tissue content of carbamate after the incubation demonstrated a similar trend.

The absorption of these carbamates through the skin followed the same pattern. Optimal absorption evidently occurred with the *n*-butyl compound, and any further increase in lipophilicity led to a decrease in absorption, contrary to the hypothesis of previous investigators.

2530. Lipids, NADPH and carbon disulphide

Wrońska-Nofer, Teresa & Sokal, J. A. (1972). The levels of the nicotinamide-adenine dinucleotides in the tissues of rabbits and rats after chronic exposure to carbon disulphide. *Int. Arch. Arbeitsmed.* **29,** 124.

Previous reports have identified several biochemical effects of carbon disulphide (CS_2) intoxication, including disturbances in serum lipid levels in rats (*Cited in F.C.T.* 1971, **9,** 745) reflected in increased blood concentrations of cholesterol and triglycerides. The workers cited above detected a change in serum nicotinamide adenine dinucleotides after prolonged exposure of animals to CS_2 and sought a possible connexion between this and the earlier findings.

Levels of the two dinucleotides in their reduced and oxidized forms (NADH, NAD^+, NADPH and $NADP^+$) were studied in the liver and blood of rats and rabbits previously exposed to CS_2 over a period of 5 months (1·5–1·8 mg CS_2/litre air for 5 hr/day on 6 days/wk). In rabbits, CS_2 exposure decreased the blood levels of the oxidized nucleotides by about 17% and of the reduced forms by about 33% compared with levels in untreated controls, and increased the hepatic NADPH concentration by about 46%. Similar effects were recorded in rats, in which there was also a tendency for NAD^+ and NADH levels in the liver to increase.

In other studies, similar conditions of exposure to CS_2 increased the serum lipid level in rats and rabbits, and in rats also increased the rate of cholesterol synthesis in the liver. Hepatic NADPH is utilized by mammals in the synthesis of fatty acids and cholesterol and there are indications that the availability of NADPH is one of the factors determining the rate of lipogenesis. The authors of this paper therefore postulate a direct link between changes in lipid metabolism and in NADPH levels following prolonged exposure to CS_2.

[The hypothesis of a connextion between serum lipids and dinucleotide levels appears to be supported by a previous finding that oral administration of nicotinic acid to rats during exposure to CS_2 prevented the rise in serum lipids that was otherwise found (*Cited in F.C.T.* 1971, **9,** 745). Changes in carbohydrate metabolism (associated with increased blood-sugar levels) 1–2 hr after CS_2 exposure may also be in keeping with this hypothesis (*ibid* 1973, **11,** 151). However, a study of the time-scale of these changes would help to elucidate the primary effect(s) of the CS_2 intoxication.]

2531. A cyanoacrylate postscript

Arthuad, L. E., Lewellen, Gale R. & Akers, W. A. (1972). The dermal toxicity of isoamyl-2-cyanoacrylate. *J. Biomed. Mater. Res.* **6,** 201.

The alkyl cyanoacrylates are used in surgery for the treatment of bleeding and in particular of severe haemorrhage from solid organs, since they polymerize and form a powerful seal on contact with water (*Cited in F.C.T.* 1973, **11,** 314). We have recently reported on evidence that, compared with several higher homologues, the methyl ester evokes more severe tissue reactions (*ibid* 1973, **11,** 317) and another paper has now appeared dealing with the effects of isoamyl 2-cyanoacrylate (IACA).

When this compound was applied to the skin of rabbits, it was found to be a moderate irritant, according to the Draize Classification, with a primary irritation score of 2·9. At the end of the 14-day observation period, the only histopathological finding was some capillary dilatation with slight infiltration of round cells in the upper dermis. On application of IACA at weekly intervals in doses of 0·05, 1·0 or 2·0 ml/kg, the tissue reaction was directly related to the concentration of IACA, but its severity was not greatly enhanced by the repeated application.

The physical properties of IACA precluded a quantitative eye irritation test, but both instillation of 0·1 ml IACA into the eye and eye contact with the vapour produced distinct irritation.

Since no significant radioactivity was detected in the epidermis or dermis of guinea-pig skin up to 14 days after the topical application of [^{14}C]IACA, it was concluded that the compound did not effectively penetrate into the skin. A lack of percutaneous absorption was also indicated by an absence of any significant increase in the radioactivity found in the urine and faeces and in the major organs, for up to 30 days after topical application of [^{14}C]IACA. The almost complete recovery of applied radioactivity from the application site was thought to be indicative of a slow rate of *in vivo* biodegradation of IACA, precluding the possibility that toxic effects could result from metabolite formation.

The results suggest that IACA has irritant and histotoxic potential, but carefully designed patch-tests in human subjects would obviously be the best method for elucidating the true hazard to man.

2532. A new component of furnace black

Neal, J. & Trieff, N. M. (1972). Isolation of an unknown carcinogenic polycyclic hydrocarbon from carbon blocks. *Hlth Lab. Sci.* **9,** 32.

Specifications such as the British Standard for food-grade channel black (*Cited in F.C.T.* 1967, **5,** 807) exclude the presence of polycyclic aromatic hydrocarbons (PAHs). Furnace blacks, however, yield benzene extracts containing PAHs which may produce tumours in animals when applied dermally or sc (*ibid* 1963, **1,** 106), although whole furnace blacks are apparently inert when ingested or inhaled (*ibid* 1963, **1,** 106).

The paper cited above describes the isolation of new PAH from eight of nine carbon blacks variously prepared by the oil- or gas-furnace process, by thermal combustion of gas or as a by-product of acetylene production. The benzene extract from these blacks contained 0·5–24·3% of the new compound, the highest level being found in a furnace black derived from aromatic oils. The compound formed flat square crystals of bright orange hue, sublimed at 165–167°C and had a molecular weight of 228–230, but (unusually for a PAH) was not fluorescent. Its ultraviolet and visible spectra and chromatographic behaviour were identical with those of material isolated from an air particulate collected from within two petrochemical plants and their immediate environs. When injected sc into mice, 6·5 mg of the compound (about 260 mg/kg) induced a 50% incidence of local fibrosarcomas after 244 days. This compared with a 100% incidence induced by the total benzene extract of one of the furnace blacks.

The molecular weight, chromatographic behaviour and spectra of the material suggested that it was identical or very similar to a compound previously isolated from coal-tar pitch and tentatively identified as acepyrene or cyclopenta[*c,d*]pyrene (Wallcave, *Envir. Sci. Technol.* 1969, **3,** 948), but further investigations would be necessary to confirm this.

2533. Oral toxicity of gentian violet

Hodge, H. C., Indra, J., Drobeck, H. P., Duprey, L. P. & Tainter, M. L. (1972). Acute oral toxicity of methylrosaniline chloride. *Toxic. appl. Pharmac.* **22,** 1.

The triphenylmethane derivative, gentian violet (methylrosaniline chloride) is used medicinally in oral doses of up to 150 mg/day for 10 days in the treatment of a number of bacterial infections. The present work was designed to throw light on the acute toxicity of the compound, since certain cases of gastro-intestinal irritation, with accompanying vomiting and diarrhoea, have been reported to follow the oral administration of the dye.

Single oral doses of gentian violet, either in solution or in powdered form, were administered to a number of animal species, and the LD_{50} values determined were 600 mg/kg for mice observed for 7 days and 250 mg/kg for rats. The approximate lethal dose for guinea-pigs, cats and rabbits ranged between 0·1 and 0·25 g/kg, while that for dogs was about 1 g/kg.

The most probable cause of death was thought to be gastro-intestinal irritation, with consequent fluid and electrolyte loss. Some dye was absorbed, but there was no significant production of methaemoglobin, at least in mice.

2534. Reactions to plastics

Jordan, W. P., Jr. & Dahl, M. V. (1972). Contact dermatitis from cellulose ester plastics. *Archs Derm.* **105,** 880.

Dermatitis associated with the wearing of new pair of spectacles has been attributed to ethylene glycol monomethyl ether acetate used to cement the nosepads to the frame (*Cited in F.C.T.* 1972, **10,** 599). Further examples of allergy to hearing aids, the frames of sunglasses, ball-point pens and shoe resins containing cellulose esters have now been investigated.

Six patients with allergic contact dermatitis attributed to cellulose ester plastics were subjected to patch testing with the various components of the plastics. This demonstrated that three of the patients were allergic to resorcinol monobenzoate, which is incorporated into plastics as an ultraviolet absorber. Two others were allergic to Solvent Yellow 3 (C.I. (1956) no. 11160) in a black dye, and one of these was also sensitive to Solvent Red 26 (C.I. (1956) no. 26120) in the same dye and to *p-tert*-butylphenol, an antioxidant. One patient reacted to the ethylene glycol monomethyl ether acetate used as a cement.

The authors point out that the ageing of cellulose plastics and their exposure to ultraviolet radiation could result in the formation of new compounds, such as 2-hydroxybenzophenone derivatives from resorcinol monobenzoate and phenyl salicylate. Thus, at least in theory, allergic contact dermatitis could be induced by a decomposition product (see p. 520). Coating articles with a polyurethane film has been shown to be a practical method of preventing some cases of dermatitis.

2535. Twenty-five years of vinyl chloride

Kramer, C. G. & Mutchler, J. E. (1972). The correlation of clinical and environmental measurements for workers exposed to vinyl chloride. *Am. ind. Hyg. Ass. J.* **33,** 19.

Prolonged exposure of rats to vinyl chloride (I) at an atmospheric level of 30,000 ppm has resulted in tumours at several sites (*Cited in F.C.T.* 1972, **10,** 601), while at 20,000 ppm liver damage and reduced spleen weight have been observed (*ibid* 1964, **2,** 266). In man, no adverse effects were found after exposure to 500 ppm for 7·5 hr (*ibid* 1970, **8,** 710) although various other studies have indicated that under certain circumstances industrial exposure

to I may be associated with angioneurosis, acro-osteolysis, neurasthenia, circulatory disturbances and hepatomegaly. Vinylidene chloride (dichloroethylene; II) has caused mortality and weight loss in animals continuously exposed for 90 days at levels of 5 and 15 ppm, and hepatic and renal damage at higher levels (*ibid* 1968, **6,** 669).

The present study is an assessment of the chronic effects of industrial exposure to I and to small amounts of II in 98 men employed for up to 25 yr in two plants using I in polymerization processes. In the case of the most heavily exposed individuals, the time-weighted average concentration (TWAC) of I in the early part of their careers was approximately 300 ppm, although overall the mean TWAC varied from 155 ppm when the study was initiated in 1950 to only 10 ppm at the end. At this latter stage, II was frequently detectable only in trace amounts, and practically always below 5 ppm.

The exposed men differed in certain aspects of medical history from employees in other departments, but none of these differences was reflected in final diagnostic categories and no exposure-related effects on general health could be identified. On the other hand, when the technique of stepwise multiple linear regression analysis was used to assess the significance of variations in 21 clinical parameters studied during regular medical examinations, five parameters—systolic and diastolic blood pressures, β-protein, bromsulphthalein and icterus index—appeared to be positively correlated with TWAC and dose and one (haemoglobin) was negatively correlated. The first four parameters also correlated with obesity and the first two and haemoglobin with age. All the regression models for these variables were significant at the $P = 0{\cdot}001$ level, except the icterus index model which was significant at the $P = 0{\cdot}03$ level. However, neither the blood pressures nor the haemoglobin values were outside normal limits, and the significance of a change in the β-protein ratio is unknown. Only the increases in icterus index and bromsulphthalein gave some cause for concern, suggesting that liver function might have been affected after prolonged exposure to I at a TWAC of approximately 300 ppm. One of the most severely affected men had a history of hepatitis prior to exposure, indicating that prolonged contact of such individuals with I may be undesirable.

The techniques used in this study are of general significance, and are offered as a possible aid in the validation of industrial hygiene standards.

[In line with this study, the American Conference of Governmental Industrial Hygienists has lowered the threshold limit value recommended for vinyl chloride from 500 to 200 ppm. That for vinylidene chloride however, remains at 200 ppm, on the basis of an unpublished 1955 study which showed no adverse effects in animals chronically exposed to an atmospheric concentration of 1000 ppm.]

NATURAL PRODUCTS

2536. A useful test for food allergy

Haddad, Z. H. & Korotzer, J. L. (1972). Immediate hypersensitivity reactions to food antigens. Detection of human IgE antibodies in vitro and exploration of immunologic mechanisms using the rat mast cell system. *J. Allergy* **49,** 210.

A study was made of the rat mast cell degranulation test using the sera of 25 patients (ranging in age from 13 months to 29 yr) who had a clear-cut history of immediate hypersensitivity reactions to milk, eggs, shellfish, other fish, walnuts, peanuts or chocolate. The

reactions were marked by symptoms ranging from urticaria to anaphylactic shock, and occurred within minutes of ingestion of the offending food.

The test was carried out by incubating the test serum with equal aliquots of a rat mast cell suspension and a specific antigen (peanut, chocolate, egg and so on, obtained in concentrations of 10 μg/ml) for 3 min. The test was deemed positive when the percentage of degranulated mast cells was more than 2·5 times higher than that seen in control situations. In three patients degranulation appeared in spite of negative skin tests, and in one patient no degranulation occurred although a skin test had been positive. In the other patients degranulation correlated with skin reactions and clinical histories. This mast cell alteration could be brought about by incubation of the serum with the appropriate food antigen or a preparation of monospecific anti-IgE. Mast cells incubated with sera of food-sensitive subjects and challenged with unrelated food antigens to which the patients had no history of sensitivity showed no significant degranulation. Complement played no part in the degranulation process, since atopic sera heated at 56°C for 30 min to destroy complement still produced degranulation; but prolongation of the heat treatment to 120 min to destroy the cell-fixing ability of IgE also abolished the degranulating ability of the sera.

2537. Garlic-induced contact dermatitis

Bleumink, E., Doeglas, H. M. G., Klokke, A. H. & Nater, J. P. (1972). Allergic contact dermatitis to garlic. *Br. J. Derm.* **87,** 6.

Several types of allergic phenomenon are associated with the consumption or handling of vegetables and fruit. In the allergic contact dermatitis suffered by some unfortunates who handle forbidden fruit—or vegetables—the responsible agents are generally of low molecular weight and soluble in acetone, ethanol or ether. A few reports of allergic contact dermatitis associated with garlic have appeared in the past 12 yr, and to these has now been added another case, in which an attempt was made to characterize the active substance.

A restaurant owner who occasionally made sauces in his establishments developed eczema of both hands. Patch tests of various sauce ingredients and other materials showed sensitivity to garlic and onion, and weakly to tulip bulbs. Aqueous, ethanolic and acetone extracts of garlic gave positive patch tests also, but of the extracts of garlic powder tested, only the aqueous extract gave a positive result. The allergen therefore appears to be hydrophilic, and it has been shown to be heat and acid labile, so it will be destroyed by domestic cooking. The greatest concentration of sensitizer was in the outer part of the bulb. The fact that the patient in the case described gave positive patch-test reactions to an acetone extract of tulip (*Tulipa gesneriana*) suggests that bulbs of the Liliaceae family and particularly those of garlic, onion and tulip, may share an allergen or contain cross-reacting allergens.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2538. Two faces of benzophenone hypersensitivity

Ramsay, D. L., Cohen, H. J. & Baer, R. L. (1972). Allergic reaction to benzophenone. Simultaneous occurrence of urticarial and contact sensitivities. *Archs Derm.* **105,** 906.

A man developed a papular eruption on the helices of both ears, and within a few months

this spread to involve light-exposed areas of the face, neck, arms and hands, which during subsequent summers showed increasing lichenification and thickening and marked erythema. During this time he was treated with topical sunscreen preparations and corticosteroids. Photopatch tests with various substances of known photoallergenic potential were negative, until he was readmitted to hospital following a severe reaction to the use of 10% sulisobenzone (2-hydroxy-4-methoxybenzophenone 5-sulphonic acid; Sungard). Scratch testing with 1% sulisobenzone provoked an immediate wheal and flare response. A similar response also followed scratch testing with 1% unsulphonated 2-hydroxy-4-methoxybenzophenone (HMBP) in butyrolactone. Patch tests with 5% sulisobenzone in water provoked a papular response after 24 and 48 hr, but this was not intensified by ultraviolet irradiation of the test site. Patch testing with 5% HMBP caused oedema and erythema after 30 min but no delayed reaction after 24 hr.

The lack of reports in the literature suggests that benzophenones have a very low sensitizing capacity. Furthermore, the simultaneous development of both immediate urticarial and delayed contact hypersensitivity to an allergen is an unusual feature. The failure of HMBP to elicit the delayed contact reaction indicates that the sulphonic acid group plays an essential role in the cell-mediated contact reaction.

2539. Favourable report on nitrilotriacetic acid

Nixon, G. A., Buehler, E. V. & Niewenhuis, R. J. (1972). Two-year rat feeding study with trisodium nitrilotriacetate and its calcium chelate. *Toxic. appl. Pharmac.* **21,** 244.

Scharpf, L. G., Jr., Ramos, F. J. & Hill, I. D. (1972). Influence of nitrilotriacetate (NTA) on the toxicity, excretion and distribution of cadmium in female rats. *Toxic. appl. Pharmac.* **22,** 186.

The proposal to use trisodium nitrilotriacetate (Na_3NTA) as a replacement for sodium tripolyphosphate as a laundry-detergent builder has led to much conflict of opinion on the potential toxicity of NTA in the environment (*Cited in F.C.T.* 1971, **9,** 888). One of the knotty problems is the possible effect of the chelating properties of NTA on tissue concentrations of heavy metals such as cadmium (Cd) or of more acceptable metals such as calcium (Ca) and zinc (Zn).

In the first study cited above, rats were fed 0·03, 0·15 or 0·5% Na_3NTA or 0·5% of its Ca chelate for up to 2 yr. All the test diets except that containing 0·03% Na_3NTA caused a greater incidence of nephritis and nephrosis, which increased in severity between 6 and 24 months. The higher dietary levels were associated with a significant rise in urinary excretion of Zn and in the Zn level of bone. Males showed a dose-related trend in survival rate whereas females did not. No evidence was found that any change in the activity of the Zn-containing enzyme, carbonic anhydrase, accompanied the alteration in Zn levels, and the increased Zn excretion may have been secondary to the kidney damage or, perhaps more probably, may have represented an increase in the absorption of dietary Zn. From these findings, it was concluded that 0·03% Na_3NTA represented the dietary no-effect level.

The second study cited reports that the 4-day mortality rate among non-pregnant rats given 64 or 80 mg $CdCl_2$/kg by oral intubation was reduced by simultaneous administration of 200 or 250 mg NTA/kg. The Cd levels of the liver and kidney of the animals given the combined treatment was lower at both 24 and 96 hr than those found in animals given $CdCl_2$ alone. In rats given Cd alone there was a gradual increase in the concentration of Cd in the kidney over the treatment period, whereas the addition of NTA induced a cumulative

increase in urinary and faecal excretion of Cd, tending to counteract this trend. Simultaneous treatment with NTA prevented the marked loss of weight that Cd alone provoked. Thus, on this evidence, NTA appears to exert a markedly protective effect against Cd intoxication by interfering with its accumulation in the tissues and increasing its excretion.

BIOCHEMICAL PHARMACOLOGY

2540. Further studies on trialkyltins and mitochondrial function

Aldridge, W. N. & Street, B. W. (1971). Oxidative phosphorylation. The relation between the specific binding of trimethyltin and triethyltin to mitochondria and their effects on various mitochondrial functions. *Biochem. J.* **124,** 221.

Selwyn, M. J., Dunnett, S. J., Philo, R. D. & Dawson, A. P. (1972). Factors affecting the inhibition of mitochondrial adenosine triphosphatase by trialkyltin compounds. *Biochem. J.* **127,** 66P.

Dawson, A. P., Dunnett, S. J. & Selwyn, M. J. (1972). Inhibitory actions of trialkyltin compounds on mitochondrial respiration. *Biochem. J.* **127,** 67P.

The process by which the body converts the energy of foodstuffs into a form that is easily assimilated, such as the phosphate-bond energy in ATP, is termed oxidative phosphorylation (*Cited in F.C.T.* 1970, **8,** 312). The mechanism of this elegant process of energy conservation has been probed using inhibitors such as trialkyltins, which bind specifically to the inner mitochondrial membrane, and much of the progress made has come from one laboratory, including an interesting hypothesis for the coupling of the respiratory chain to ATP synthesis (*ibid* 1970, **8,** 242). Another report from the same laboratory (first paper cited above) examines the inhibition of oxidative phosphorylation that results from the binding of triethyltin (TET) and trimethyltin (TMT) to a site (known as binding site 1) in mitochondria, in the light of current theories on the coupling of the respiratory chain to the formation of ATP.

Added to an *in vitro* phosphorylating system prepared from rat liver, TET and TMT inhibited pyruvate and succinate oxidation, both being linked to ATP synthesis. Up to about 50% inhibition, the ratio of inhibition to binding sites complexed was about 10 times greater than would have been expected on the assumption that one molecule of inhibitor was bound by one site. Complexing of 20–30% of binding site 1 was thus sufficient to cause virtually complete inhibition. Oxidation of ascorbate (associated with that of reduced cytochrome *c* and with phosphorylation of one molecule of ADP), ATP hydrolysis and oxygen uptake, were all inversely proportional to the amount of binding site 1 complexed, indicating that this site is completely involved in phosphorylation.

These results are discussed in the light of numerous theories regarding the effects of the trialkyltins on mitochondrial membrane structure. Since site 1 does not have to be saturated for complete inhibition to occur, it appears that each respiratory chain must be associated with more than one binding site—in fact the results indicate complexes of ten clustered loci, only one of which has to be bound with TET or TMT for full inhibition of phosphorylation to result. Much work is still required, however, to integrate the results and theories from all sources.

The above report also implies that trialkyltins have a potent inhibitory effect on 2,4-dinitrophenol-stimulated ATPase. The second paper cited above demonstrates this effect in rat-liver mitochondria. Magnesium ions were required, sensitivity to inhibition decreasing in an exponential fashion away from optimum magnesium concentrations. The situation in whole mitochondria was complicated by pH changes resulting from movements of inorganic ions triggered by the inhibitor. Where inorganic phosphate was also present, the effectiveness of these inhibitors was decreased, probably because of phosphate-complex formation. The fact that both trialkyltins and oligomycin inhibit the ATPase of fragmented mitochondria and the finding that tributyltin only inhibited soluble ATPase in the presence of a factor that also confers oligomycin-sensitivity on the ATPase indicate that the site of action of trialkyltins is the same as that of oligomycin.

A short report (also cited above) from the same laboratory suggests that the inhibitory effect of trialkyltins on 2,4-dinitrophenol-stimulated respiration is correlated with the ability of these compounds to catalyse Cl^-–OH^- ion exchange. Such exchange may allow rapid pH equilibration on either side of the mitochondrial membrane (as opposed to the action of uncoupling agents which cause proton uptake) and the pH alteration would lower the activity of the respiratory enzymes.

Fd Cosmet. Toxicol. Vol. 11, pp. 525–526. Pergamon Press 1973. Printed in Great Britain

FORTHCOMING PAPERS

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

Long-term toxicity studies of erythrosine. I. Effects in rats and dogs. By W. H. Hansen, R. E. Zwickey, J. B. Brouwer and O. G. Fitzhugh.

Long-term toxicity studies of erythrosine. II. Effects on haematology and thyroxine and protein-bound iodine in rats. By W. H. Hansen, K. J. Davis, S. L. Graham, Carleene H. Perry and K. H. Jacobson.

Modification of acute toxicity of mutagenic and carcinogenic chemicals in the mouse by prefeeding with antioxidants. By R. B. Cumming and Marva F. Walton.

Metabolism of the phenolic antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354). I. Excretion and tissue distribution in man, rat and dog. By J. W. Daniel, T. Green and P. J. Phillips.

Metabolism of the phenolic antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354). II. Biotransformation in man, rat and dog. By J. W. Daniel, T. Green and P. J. Phillips.

Metabolism of the phenolic antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354). III. The metabolism in rats of the major autoxidation product, 2,6-di-*tert*-butyl-*p*-benzoquinone. By J. W. Daniel, T. Green and P. J. Phillips.

Studies on carrageenan and large-bowel ulceration in mammals. By P. Grasso, M. Sharratt, F. M. B. Carpanini and S. D. Gangolli.

Intestinal effects of carrageenans in the rhesus monkey (*Macaca mulatta*). By K.-F. Benitz, L. Golberg and F. Coulston.

Sensitivity of the guinea-pig to raw soya bean in the diet. By J. R. Patten, J. A. Patten and H. Pope, II.

Toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. By J. B. Greig, Glenys Jones, W. H. Butler and J. M. Barnes.

The effects of long-term ingestion of methimazole on the thyroids of rats. By N. V. Owen, H. M. Worth and G. F. Kiplinger.

Is oestrogenic activity present in hops? By Catherine Fenselau and P. Talalay.

Excretion and metabolism of orally administered aflatoxin B_1 by rhesus monkeys. By J. I. Dalezios, D. P. H. Hsieh and G. N. Wogan.

Inhibition of alcohol and lactic dehydrogenases by patulin and penicillic acid *in vitro*. By S. H. Ashoor and F. S. Chu.

Hexachlorophene concentrations in blood associated with the use of products containing hexachlorophene. By A. G. Ulsamer, F. N. Marzulli and R. W. Coen.

Effect of hexachlorophene on the rat brain during ontogenesis. By L. Nieminen, K. Bjondahl and M. Möttönen.

Study of long-term percutaneous toxicity and carcinogenicity of hair dyes (oxidizing dyes) in rats. By H. J. Kinkel and S. Holzmann.

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 (female authors may use one given name) 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. I. 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:

e.g. 1943a, 1943b or (1943a, b).

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

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on *separate* sheets. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

▲ △ ■ □ ● ○ ⊙ × ◇

Photographs and photomicrographs should be submitted unmounted and on glossy paper. When colour plates are to be printed, payment for blockmaking is the responsibility of the author.

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

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

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

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

Reprints. Fifty reprints of the paper will be supplied free of charge. An order form for additional reprints will accompany the proofs.

Frequency. The Journal will be published bi-monthly.

Printed in Great Britain by A. Wheaton & Co., Exeter