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EFFECTS OF PROLONGED ETHYLENE THIOUREA INGESTION ON THE THYROID OF THE RAT

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(*Received* 17 *March* 1975)

Abstract—Male and female Charles River rats, approximately 5 wk old, were fed ethylene thiourea (ETU) for 2 yr at levels of 0, 5, 25, 125, 250 or 500 ppm in the diet. Body weights, the weights of the thyroid and other organs, thyroidal uptake of ^{131}I, haematology and histology were the criteria studied. ETU was found to be a thyroid carcinogen for the rat at the 250 and 500 ppm dietary levels and a thyroid tumorigen at the 125 ppm level; it caused slight thyroid hyperplasia at the 5 and 25 ppm feeding levels. Thyroid hyperplasia was not noticeably reversible in those rats in each group that were changed to control diet after 66 wk on ETU diets. ETU ingestion at the 5 and 25 ppm levels was not biologically deleterious to the rat.

INTRODUCTION

Dithiocarbamates have been used as fungicides against a variety of pathogenic plant fungi. Smith, Finnegan, Larson, Sahyoun, Dreyfuss & Haag (1953) reported that mild thyroid hyperplasia occurred in rats fed the ethylenebisdithiocarbamate fungicides throughout life. These compounds are unstable and one of the degradation products is ethylene thiourea (ETU). Ludwig & Thorn (1958) suggested a possible degradation scheme for metallic derivatives of the ethylenebisdithiocarbamates (Fig. 1), speculating that ethylenebisdithiocarbamic acid readily forms ETU under highly alkaline conditions (pH 10·5) and that ETU obtained under these conditions may be formed from ethylenethiuram monosulphide by the loss of a molecule of carbon disulphide.

The only toxicological study of ETU reported in the literature until recent years was that of Seifter & Ehrich (1948). These investigators fed ETU to weanling rats at a dietary level of 0·1% for 8 days and noted decreased growth, increased thyroid weight and marked thyroid hyperplasia.

Interest in ETU was revived when Innes, Ulland, Valerio, Petrucelli, Fishbein, Hart, Pallotta, Bates, Falk, Gart, Klein, Mitchell & Peters (1969) reported that this compound was tumorigenic for the liver. In view of the very large number of compounds involved in what was intended to be a preliminary study, a standardized protocol was essential, and this made no provision for examination of the thyroid glands of any animals, including those given ETU. In a short-term feeding study using male Osborne-Mendel rats, thyroid weights were found to be increased, ^{131}I uptakes were decreased and, at high doses (500 and 750 ppm), thyroid hyperplasia was observed (Graham & Hansen, 1972). In another study, in which rats were fed a diet containing 350 or 175 ppm ETU for 18 months followed by a control diet for 6 months, it was concluded that ETU has an action like that of a number of other thio compounds that cause thyroid carcinomas and indirectly affect the liver (Ulland, Weisburger, Weisburger, Rice & Cypher, 1972).

We reported previously the results of a study in which Charles River rats were fed ETU for 12 months (Graham, Hansen, Davis & Perry, 1973). Microscopic examination of thyroids from the rats fed dietary levels of 125 ppm or more revealed nodular hyperplasia, while thyroid carcinomas were found in some rats fed 250 or 500 ppm. This report is a continuation of that study and described the effects on the thyroid glands of rats after 18 and 24 months of ETU administration.

EXPERIMENTAL

Five groups of 68 male and 68 female Charles River rats (caesarean-derived) were started when approximately 5 wk old on diets containing ETU at a level of 5, 25, 125, 250 or 500 ppm. The diets were prepared every 2 wk by mixing appropriate amounts of ETU (Lot No. 4876, K & K Laboratories, Plainview, N.Y.) into ground Purina Chow (Ralston Purina Co., St. Louis, Mo.). Feed cups were filled weekly, and the feed from each mixing batch to be used in the second week was stored in closed containers at 4·5°C until needed. Assays showed that no loss of ETU occurred within the 1-wk period during which the diet was in the feed cups. Control rats were fed the basic diet of Purina Chow. All rats were housed individually and food and water were provided *ad lib.* Body weights and food consumption were recorded every 7 days.

At the end of the 18- and 24-month feeding periods, five females and five males from each group were given an ip injection of 0·2 ml physiological saline

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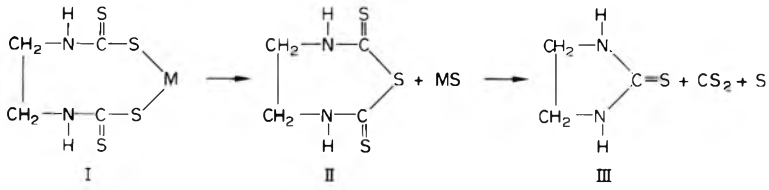

Fig. 1. Degradation scheme for metallic derivatives of the ethylenebisdithiocarbamates: I, zineb (where M = zinc) or maneb (where M = manganese); II, ethylenethiuram monosulphide; III, ethylene thiourea (ETU).

prepared to contain approximately 5 μCi ^{131}I. The rats were fasted for 24 hr and were then given a barbiturate overdose. The thyroids, heart, liver, kidneys and spleen and, in the case of the males, the testes were removed and weighed. The radioactivities of individually weighed thyroid pairs were determined with a gamma well counter. Reference standards were prepared at the time of injection and all counts were compared with the standard as an average of three successive determinations of counts/min. The uptake was determined after conventional corrections were made for radioactive decay.

After 17 and 22 months, blood samples were collected from the tail vein of ten male and ten female rats fed each dietary level for determinations of haemoglobin and haemotocrit values and for red blood cell, leucocyte and differential leucocyte counts.

Three male and three female rats from each test group were returned to the control diet at wk 66 and kept on this for the rest of the 2-yr period to check for reversibility of toxicity. These rats were selected by palpating the thyroids of several rats from each test level and taking only those with enlarged glands.

Tissues were removed for gross evaluation from all the animals killed after treatment for 18 or 24 months, and paraffin sections of the formalin-fixed thyroids were stained with haematoxylin and eosin and examined by light microscopy. In addition, similarly stained paraffin sections of formalin-fixed gross lesions (except middle-ear infections) and other selected tissues from rats that died or were killed during yr 2 (total, 432 rats) were transferred to the Environmental Protection Agency for microscopic examination.

All data were compiled and tested for significance by the two-tailed Student's t test.

RESULTS

Body weights

Table 1 shows the effect of ETU ingestion on the body weights of rats. The weights of males and females fed ETU at the 500 ppm dietary level were significantly decreased from control values at both 18 months ($P < 0.001$) and 24 months ($P < 0.01$).

Organ-to-body weight ratios

At 18 months. Compared with the control ratios, significant ($P < 0.05$) increases were recorded after ETU administration for 18 months for the liver-to-body weight ratio in males fed the 250 ppm diet and for the thyroid-to-body weight ratios in males fed 500 ppm and females fed 250 ppm (Table 2). Although ten animals of each sex from each group had been killed at 2, 6 and 12 months (Graham *et al.* 1973), it was necessary to reduce the number to five in each case at 18 months because of decreasing numbers of animals; some values in Table 2 are, therefore, biologically different from the control values but are not statistically different because of large variations and small numbers of animals. There were no differences from control values in the organ-to-body weight ratios of the kidneys, spleen, heart and testes in males or of the liver, kidneys, spleen and heart in females.

At 24 months. Table 3 gives the organ-to-body weight ratios for rats fed ETU for 24 months. In males fed the 500 ppm diet, ratios for the heart and thyroid were significantly higher ($P < 0.01$ and < 0.05, respectively) than those of the controls. In females, statistically significant decreases in liver-to-body weight ratios were seen in the groups fed diet containing 5 or 25 ppm ETU ($P < 0.05$), and thyroid-to-body weight ratios were significantly increased ($P < 0.01$) in the groups fed the 250 or 500 ppm levels.

Uptake of radioactive iodine

Uptakes of iodine (expressed as counts/min/mg tissue) 24 hr after injection are shown in Figs 2 & 3. The histograms show the percentage change from control values.

In male rats fed 25 or 125 ppm ETU for 18 months, the ^{131}I uptake showed statistically significant increases of 64 and 52%, respectively, compared with

Table 1. *Mean body weights of Charles River rats fed 0–500 ppm ETU in the diet for 18 or 24 months*

Dietary level (ppm)	Mean body weight (g) at month	
	18	24
	Males	
0	721 ± 19 (32)	710 ± 25 (15)
5	759 ± 13 (35)	741 ± 20 (20)
25	695 ± 14 (35)	669 ± 21 (22)
125	725 ± 16 (34)	680 ± 28 (19)
250	687 ± 16 (26)	682 ± 41 (12)
500	606 ± 27***(23)	526 ± 56**(6)
	Females	
0	509 ± 18 (34)	564 ± 35 (17)
5	507 ± 18 (34)	556 ± 30 (19)
25	438 ± 15 (34)	496 ± 24 (22)
125	439 ± 16 (35)	516 ± 34 (20)
250	473 ± 12 (32)	502 ± 26 (21)
500	427 ± 13***(32)	415 ± 20**(14)

Values are expressed as the mean ± SEM for the numbers of rats indicated in parentheses. Those marked with asterisks differ significantly (Student's t test) from the control: **$P < 0.01$; ***$P < 0.001$.

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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

*These items have been contributed by the staff of the British Industrial Biological Research Association. Comments on them will be welcomed and should be sent to the Assistant Editor at BIBRA.

Table 2. *Mean organ-to-body weight ratios of Charles River rats fed 0–500 ppm ETU for 18 months*

Dietary level (ppm)	Organ-to-body weight ratio (g/kg)					
	Liver	Kidney	Spleen	Heart	Testes	Thyroid†
			Males			
0	23·30 ± 1·81	5·86 ± 0·64	1·46 ± 0·22	2·50 ± 0·15	5·28 ± 0·31	49·6 ± 3·7
5	28·34 ± 2·49	6·13 ± 0·64	1·49 ± 0·13	2·76 ± 0·30	5·58 ± 0·30	42·0 ± 3·5
25	25·59 ± 0·99	5·54 ± 0·37	1·41 ± 0·16	3·04 ± 0·41	5·93 ± 0·19	45·4 ± 3·7
125	24·43 ± 1·58	5·06 ± 0·18	1·28 ± 0·09	2·54 ± 0·19	5·22 ± 0·39	48·0 ± 2·7
250	28·36 ± 0·83*	6·71 ± 0·21	1·49 ± 0·08	2·94 ± 0·32	5·88 ± 0·47	155·8 ± 44·8
500	27·72 ± 1·52	5·70 ± 0·40	1·24 ± 0·17	2·49 ± 0·16	5·99 ± 0·22	728·6 ± 155·3*
			Females			
0	24·56 ± 0·80	5·60 ± 0·18	1·62 ± 0·16	2·71 ± 0·19	—	76·2 ± 9·7
5	21·72 ± 1·23	5·14 ± 0·31	1·49 ± 0·15	2·60 ± 0·21	—	58·2 ± 5·7
25	24·58 ± 1·50	4·97 ± 0·29	1·30 ± 0·06	2·51 ± 0·16	—	56·0 ± 8·1
125	28·60 ± 3·09	5·65 ± 0·58	1·48 ± 0·10	2·77 ± 0·22	—	76·2 ± 7·2
250	25·70 ± 1·18	5·94 ± 0·33	1·46 ± 0·11	2·81 ± 0·11	—	122·4 ± 13·1*
500	31·09 ± 2·43	5·90 ± 0·47	1·78 ± 0·47	2·98 ± 0·47	—	1894·4 ± 1182·3

†Thyroid ratios are expressed in mg/kg.
Values are means ± SEM for groups of five rats and those marked with an asterisk differ significantly (Student's *t* test) from the control value: $*P < 0{\cdot}05$.

Table 3. *Mean organ-to-body weight ratios of Charles River rats fed 0–500 ppm ETU for 24 months*

Dietary level (ppm)	No. of rats	Organ-to-body weight ratio (g/kg)					
		Liver	Kidney	Spleen	Heart	Testes	Thyroid†
				Males			
0	15	24·95 ± 0·80	6·56 ± 0·36	1·97 ± 0·22	2·69 ± 0·08	6·00 ± 0·57	67·2 ± 4·4
5	20	24·27 ± 0·92	6·96 ± 0·59	1·64 ± 0·10	2·66 ± 0·09	4·99 ± 0·20	58·8 ± 3·2
25	21	25·34 ± 0·82	7·38 ± 0·57	1·73 ± 0·10	2·83 ± 0·08	5·90 ± 0·27	67·0 ± 12·4
125	19	30·76 ± 3·73	7·09 ± 0·58	2·50 ± 0·43	3·06 ± 0·19	5·46 ± 0·20	147·4 ± 65·3
250	11	25·01 ± 1·35	6·90 ± 0·70	1·62 ± 0·30	2·80 ± 0·13	5·08 ± 0·51	209·4 ± 64·6
500	5	26·17 ± 3·01	6·90 ± 0·71	1·56 ± 0·34	3·28 ± 0·24**	5·65 ± 0·69	1028·4 ± 155·6*
				Females			
0	17	28·71 ± 1·55	5·75 ± 0·38	1·87 ± 0·31	2·62 ± 0·20	—	74·3 ± 7·5
5	19	24·78 ± 1·16*	5·72 ± 0·37	2·23 ± 1·30	2·73 ± 0·15	—	65·4 ± 4·4
25	22	24·74 ± 0·82*	5·94 ± 0·06	2·50 ± 0·68	2·86 ± 0·12	—	65·9 ± 3·3
125	18	25·33 ± 1·63	5·71 ± 0·36	1·33 ± 0·02	2·59 ± 0·11	—	84·9 ± 5·6
250	21	30·07 ± 1·44	6·60 ± 0·72	1·74 ± 0·15	2·91 ± 0·21	—	182·7 ± 31·2**
500	13	30·75 ± 4·07	6·59 ± 0·29	1·71 ± 0·08	3·03 ± 0·12	–	1279·5 ± 357·6**

†Thyroid ratios are expressed in mg/kg.
Values are means ± SEM for the numbers of rats stated and those marked with asterisks differ significantly (Student's *t* test) from the control value: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

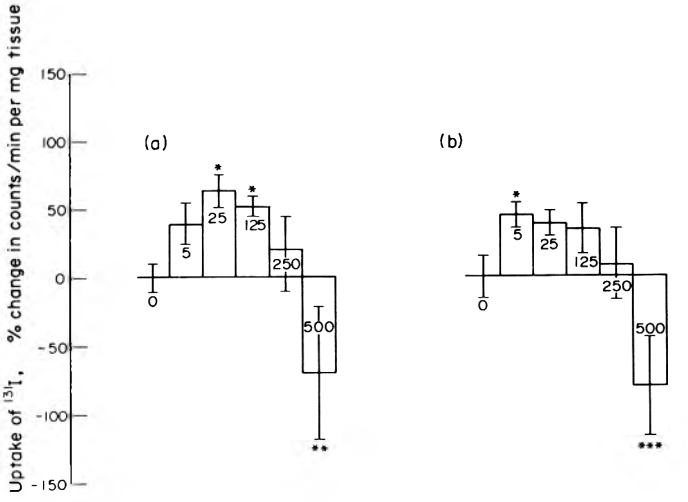

Fig. 2. Mean uptake of ^{131}I by the thyroids of groups of five male rats fed 0–500 ppm ETU for (a) 18 or (b) 24 months. Vertical bars represent the SEM and asterisks indicate results differing significantly from the control: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

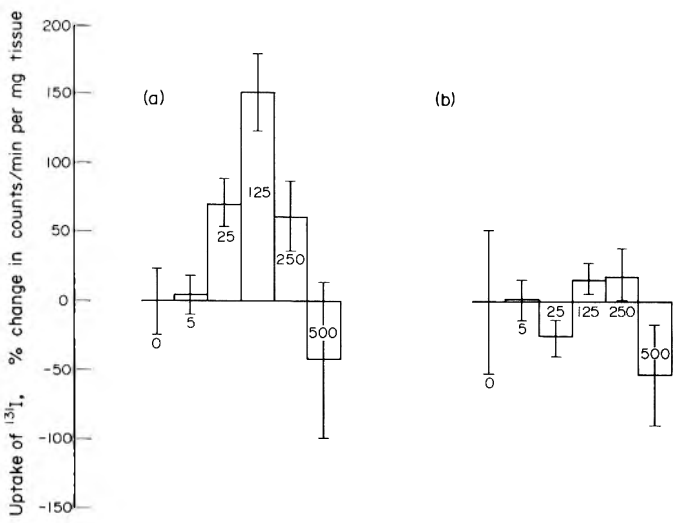

Fig. 3. Mean uptake of ^{131}I by the thyroids of groups of five female rats fed 0–500 ppm ETU for (a) 18 or (b) 24 months. Vertical bars represent the SEM.

control values (Fig. 2). Male rats fed at the 500 ppm level showed statistically significant decreases in uptake, the value being 70% lower than the control value. After 24 months, male rats fed the 5 ppm ETU diet showed a statistically significant increase in ^{131}I uptake, and there was a statistically significant decrease in uptake (80% lower than the control value) in those fed 500 ppm.

Figure 3 represents the ^{131}I uptake in the thyroids of female rats fed ETU in their diets for 18 or 24 months. There were no statistically significant differences in uptake in the females fed any level of ETU. Because of large variability and low numbers of animals at these time periods, values which were biologically prominent were not statistically significant.

Haematology

No effects were observed on haemoglobin concentrations or haematocrits, or in the red cell, leucocyte or different leucocyte counts of male or female rats fed ETU for 17 or 22 months.

Histological examination

Table 4 summarizes the major histopathological findings in Charles River rats fed ETU for 12–24 months. Grossly normal tissues of a number of animals were sectioned both to establish ranges of normal histology in this group of rats, and to look for subtle evidence of ETU toxicity. The thyroid had been shown to be the target tissue in shorter-term feeding studies and therefore attempts were made to examine thyroid sections from as many rats as possible. Lung sections from most rats were prepared so that particular effort could be made to examine them for possible metastases in each animal showing any form of gross tumour. Salivary lymph node sections were carefully examined for any spread of thyroid tumour in most

Table 4. *Summary of major pathological data for Charles River rats fed 0–500 ppm ETU for 12–24 months*

Parameter	Data for rats given ETU in the diet at a level (ppm) of					
	0	5	25	125	250	500
No. of rats/group						
On test at 1 yr	72	75	73	73	69	70
Sacrificed for biochemistry at 18 months	10	10	10	10	10	10
Two-year survivors	32	39	43	37	32	18
Pathological lesions						
Encephalitis/gliosis	0	2	0	1	1	0
Pituitary carcinoma	0	2	0	0	0	1
Pituitary adenoma	34	28	28	37	29	27
Pituitary hyperplasia	4	5	8	3	7	3
Cataracts/keratitis	2	1	0	2	6	12
Sinusitis/rhinitis	1	0	0	1	0	0
Adenitis, salivary or lachrymal gland	0	0	1	0	0	4
Thyroid carcinoma/adenocarcinoma	2	2	1	2	16	62
Thyroid adenoma(s)	2	—	5	1	21	3
Thyroid hyperplasia	4	20	41	44	27	3
Parathyroid hyperplasia	6	11	8	2	3	0

Table 4 (*Continued*)

Parameter	Data for rats given ETU in the diet at a level (ppm) of					
	0	5	25	125	250	500
Tracheitis	14	18	1	3	0	6
Pulmonary congestion/oedema	13	10	3	14	13	19
Pneumonia	3	8	9	6	6	20
Pulmonary adenoma/carcinoma	1	0	0	0	0	1
Peribronchial lymphocytic cuffing/atelectasis	7	1	2	4	3	4
Myocardial fibrosis/pericarditis	3	4	5	9	4	6
Hepatic carcinoma	1	0	0	0	0	0
Hepatoma	1	1	1	2	1	5
Hepatic hyperplasia	1	4	5	3	2	0
Cholangiofibrosis	1	1	1	3	1	1
Hepatic necrosis/hepatitis	4	4	1	4	3	3
Hepatic fatty metamorphosis	5	1	3	8	7	8
Gastritis/ulcers	4	3	0	2	3	6
Pancreatic duct adenoma	0	0	0	0	0	1
Pancreatic islet cell adenoma	2	0	0	0	0	0
Adrenal carcinoma	2	1	1	0	1	0
Adrenal adenoma	0	0	0	2	0	2
Adrenal haemocysts/hyperplasia	26	26	40	34	33	33
Splenic hyperplasia/myelosis/haemosiderosis	15	5	5	3	3	21
Intestinal adenocarcinoma	0	0	0	0	1	0
Renal carcinoma/liposarcoma	1	0	0	0	1	1
Nephritis/nephrosis	22	23	20	24	19	10
Urinary cystitis	1	3	3	4	3	2
Urinary bladder carcinoma	1	0	0	0	0	0
Prostatitis	5	3	2	9	4	8
Prostatic fibroadenoma	0	0	0	1	0	0
Testicular atrophy	4	4	5	3	4	8
Interstitial-cell tumour	1	0	1	1	0	0
Preputial gland adenoma	1	0	0	0	0	0
Ovarian carcinoma	0	1	0	0	0	0
Uterine/endometrial sarcoma	0	0	0	0	1	0
Uterine polyp	1	2	0	1	2	1
Metritis	2	1	3	1	0	1
Mesothelioma	1	0	1	1	0	0
Peritonitis	0	0	1	0	0	0
Septicaemia	0	0	0	1	1	0
Mesenteric/seatitis	0	1	3	0	2	2
Mesenteric arteritis/arteritis	0	4	2	2	0	2
Lipoma	0	0	0	0	0	1
Lymphosarcoma	3	3	3	5	5	1
Fibrosarcoma	0	0	5	4	5	3
Fibroma	0	1	0	0	0	0
Squamous/epidermoid carcinoma	2	1	0	2	5	2
Epidermoid cyst	1	1	2	0	1	1
Nose papilloma	0	0	0	0	1	0
Carcinoma/adenocarcinoma	0	2	0	2	0	0
Mammary adenocarcinoma	2	14	4	2	5	0
Mammary fibroadenoma	18	10	11	15	20	12
Mammary cystic hyperplasia	4	3	5	3	5	2
Osteogenic sarcoma	1	1	0	0	0	0
Bone marrow hyperplasia	0	1	0	1	0	5
Foot pad granuloma	7	15	5	9	4	7
Abscess/granuloma	1	5	3	2	0	6
Middle ear infection (not sectioned)	9	7	3	6	9	5
No. of rats/group						
With malignant tumours	15	23	13	16	31	63
With benign tumours only	36	26	32	38	30	5
Without neoplasia	21	26	28	19	8	2
Mean no. of tumour types/rat	0·986	0·900	0·836	1·041	1·608	1·714

rats showing thyroid enlargement; however, thyroid-tumour metastasis to these lymph nodes was found in only one case. No spread of thyroid tumour into the tongue was ever noted. Thyroid-tumour metastases to the lungs were seen in a number of the rats fed the highest level of ETU. Thyroid tumours in rats fed the highest ETU level were generally follicular adenocarcinomas, and there was the impression that benign tumours in rats fed the lowest level tended to be composed of solid sheets of neoplastic cells. Thin colloid could usually be demonstrated in some of the follicles in metastatic lesions in the lung. Thyroid hyperplasia was graded as slight, moderate or marked (Seifter & Ehrich, 1948) in the individual descriptions, but all grades were combined for purposes of tabulation. Generally the hyperplasia observed at the lower dose levels was slight but the thyroids were still distinguishable from control thyroids by lobulation, follicular size and uniformity, height of follicular epithelium, colloid staining, keratinization of follicles, and/or general size. Mean thyroid weights were 45 mg for control males killed at the end of the experiment and 37 mg for control females. Mean thyroid weights for both sexes given the 5 and 25 ppm ETU diets were slightly less than control weights; in animals given higher ETU levels, weights were increased. Thyroidectomy cells had been observed in the pituitary glands of some of the rats fed at the highest dose level and killed at the end of yr 1, but were seen in only a few of the rats fed ETU for longer terms and were not a prominent change. Rapidly growing immature male rats frequently show a grade 1 thyroid hyperplasia which can be masked by inanition or other slowing of the growth rate.

Histomorphological changes in the pituitary were expected to accompany treatment effects in the thyroid; however, no dose-related hyperplasia or neoplasia of the pituitary was apparent. The incidence of cataracts/keratitis was increased in rats fed the highest ETU level, an increase that may have been due both to a slightly adverse effect of treatment and to the greater number of eyes examined (59 from the high-dose group; 20 from the control group). Cataracts were usually minimal and occasionally unilateral. Parathyroid hyperplasia, as usual, was related to nephrosis. Both were decreased in rats fed 500 ppm ETU. Pneumonia was slightly more common in rats fed the highest dose level; it was surprising that more of this group had not strangled from obstruction of the trachea by enlarged thyroids. There was a slight increase in hepatoma incidence in rats fed the high dose level, compared with the incidence in controls.

Gastritis/ulcers were probably primarily an indicator of terminal stress, as were the adrenal changes of cortical haemocysts, cystic degeneration and hyperplasia. The slightly increased incidence of testicular atrophy in males fed the 500 ppm dose level may also be related to toxicity/inanition. Testicular atrophic changes were frequently slight to moderate in degree, and unilateral. Lymphosarcoma incidence did not show any significant dose-relationship. Animals are usually fairly healthy when lymphosarcoma develops, and perhaps the poor condition of the rats given the highest dose level also accounted for the decreased incidence of mammary tumours. Except for middle-ear infections, pathology data listed in Table 4 are based on histological diagnoses. For the totals given in Table 4, rats with multiple mammary tumours are each reported only as one rat with mammary tumour and are listed according to the most malignant lesion found in each.

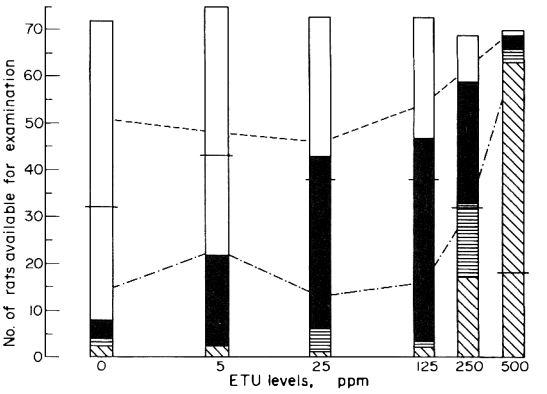

Fig. 4. Incidence of neoplasia in rats fed 0–500 ppm ETU for 1–2 yr: ■, hyperplasia; ▤, thyroid adenomas; ▨, thyroid carcinomas; ——, survivors; – – –, total tumour incidence; –·–·–, incidence of malignant tumours.

Figure 4 summarizes the neoplasia in rats fed ETU for 1–2 yr. Although the incidence of slight thyroid hyperplasia was increased in all the test groups, this effect was offset by the beneficial effects of a slightly lower malignant-tumour incidence and a slightly increased survival at the 25 ppm level.

DISCUSSION

The data show that ETU causes statistically significant decreases in body weight in both male and female Charles River rats exposed to 500 ppm in the diet. Increases in thyroid-to-body weight ratios were apparent at levels of 250 and 500 ppm in both male and female rats. Previous data from the 12-month study (Graham *et al.* 1973) showed significant decreases in body weight and increases in thyroid-to-body weight ratios in male and female Charles River rats fed levels of 250 and 500 ppm ETU in the diet. Uptake of ^{131}I, expressed as counts/min/mg tissue, was significantly decreased in male rats fed 500 ppm ETU in the diet for 18 or 24 months, as it had been at 12 months. Thyroids of females fed the three high dose levels were hypofunctioning at 6 months and hyperfunctioning at 12 months. At 24 months, they were similar to the control state and females fed the 500 ppm level had a decreased thyroid function somewhat like that of males at the same dose level.

Although these rats were purchased rather than obtained from our own FDA stock and therefore were larger than our usual starting animals (approximately 95 g), they were still well within the usual period of growth-related thyroid hyperplasia. Another factor thought to influence thyroid neoplasia was the amount of ETU consumed daily. Male rats on the 500 ppm feeding level consumed an average of 52 mg/kg/day during wk 1 of the study. ETU consumption figures for later weeks were (in mg/kg/day); 46 in wk 4, 27 in wk 13 and 12·7 in wk 104. Both the age, susceptibility and actual amount consumed each day were probably significant factors in the relative lack

of tumour progression in either incidence or malignancy after the end of yr 1 of the test. Male rats fed the 500 ppm level had each consumed an average of approximately 7·5 g ETU by the end of wk 104 of the study. The dose-related increase in thyroid-tumour incidence at the higher ETU feeding levels resulted in an increase in the average number of tumours per animal despite the deficit in mammary tumours at the highest dose level. Thyroid tumours in these rats, as in man, are relatively non-fatal neoplasms. Male rats and human females are the more susceptible sexes for thyroid tumours in the respective species. No indication of morphological reversibility was noted in test rats returned to the control diet from wk 66 to the termination of the test.

ETU is a thyroid carcinogen for Charles River-CD rats when fed at levels of 250 and 500 ppm in the diet for 2 yr. As noted in Table 4, the incidence of thyroid tumours in rats at the 125 ppm feeding level did not differ from that of the controls during yr 2; however, when the rats developing thyroid tumours during yr 1 were added to these figures, there was an increase in thyroid tumours in the rats fed 125 ppm ETU compared with controls. The tumours also arose earlier; therefore ETU behaved as a weak thyroid tumorigen at the 125 ppm feeding level in this study. We do not consider the 25 and 5 ppm ETU levels to have been tumorigenic in this study, although both levels increased the incidence of histological thyroid hyperplasia.

Data to date on ETU-induced thyroid neoplasia in the rat support the view that these tumours are the result of excessive pharmacological stimulation, i.e. they occur because of excessive endocrine-organ stimulation and not because of the reaction of one molecule of carcinogen with one cell to initiate neoplasia. Data in support of this view are the lack of thyroid tumours at yr 1 in rats given the 5 or 25 ppm dose levels, the sharp dose-related increase in tumour incidence after 1 yr in rats fed the 125, 250 or 500 ppm dose levels and confirmed after 2 yr in rats fed the 250 and 500 ppm dose levels, and the lack of a significant increase in thyroid neoplasia after yr 1, when food intake had decreased, in rats fed the 125 ppm dose level.

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TISSUE DISTRIBUTION, EXCRETION AND BIOLOGICAL EFFECTS OF [^{14}C]TETRACHLORODIBENZO-*p*-DIOXIN IN RATS

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Abstract—When Sprague–Dawley rats weighing 200 g were intubated intragastrically with ^{14}C-labelled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a dose of 50 μg/kg body weight, 50% of the animals died within 25 days. Over 56% of the radioactivity was eliminated, mainly by the alimentary route, during the initial 21 days, 25% being accounted for during the first 3 days. The total amount of radioactivity in the urine was 4·5% of the total dose, the highest daily levels being excreted toward the end of the experiment. A large percentage of the radioactivity remaining in the body was localized in the liver and of this, over 90% was within the microsomal fraction. The major morphological changes in these rats were a marked liver hypertrophy and thymic regression.

INTRODUCTION

The chlorodibenzo-*p*-dioxins have recently been reported to be widespread and lethal environmental contaminants. Research on the dioxins has shown them to be capable of producing chloracne (Hofman, 1957), hepatocellular changes (Norback & Allen, 1969), gastric mucosal hyperplasia and ulceration (Allen & Carstens, 1967), altered capillary permeability (Allen, 1964), retarded sexual development (Allen & Lalich, 1962), inhibited immunological responses (Vos, Moore & Zinkl, 1973), and embryotoxicity (Neubert, Zens, Rothenwallner & Merker, 1973). In fact, tetrachlorodibenzo-*p*-dioxin has been reported to be one of the most potent teratogens known (Courtney & Moore, 1971).

One of the major questions has been the source of these dioxins. Higginbotham, Huang, Firestone, Verrett, Ress & Campbell (1968) pyrolysed commercially available chlorophenols and obtained chlorodibenzo-*p*-dioxins. The most toxic pyrolysis product, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from 2,4,5-trichlorophenol. Hexa-, hepta- and octachlorodibenzo-*p*-dioxins were obtained from pentachlorophenols. Firestone, Ress, Brown, Barron & Damico (1972) reported the presence of dioxins and chlorinated dibenzofurans in a wide variety of commercial chlorophenols. A major source of environmental contamination by the dioxins has been the use of the defoliant, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). It is inherent in the production of 2,4,5-T that a variable amount of the dioxins is produced.

The study reported here is part of a series of experiments initiated to clarify the absorption, metabolism, tissue distribution and excretion of TCDD in various mammalian species.

EXPERIMENTAL

Eighty male Sprague–Dawley rats weighing 200 g were divided into two equal groups and given by gastric intubation either [^{14}C]TCDD (0·46 mCi/mg) in a dose of 50 μg/kg body weight, suspended in corn oil, or a similar volume of unmodified corn oil. These animals were placed in metabolism cages and 24-hr samples of urine and faeces were collected. Five rats from each group were sacrificed at 1, 3, 5, 7, 14 and 21 days. The remaining 20 animals (ten controls and ten test animals) were continued on the experiment until 50% of the experimental group had died. Prior to being killed by decapitation, the animals were deprived of food overnight and weighed. Blood was collected from rats killed on day 21; a complete blood count was made and serum levels of protein, total lipids, cholesterol and triglycerides were determined (Allen & Ahlgren, 1968).

Tissues from all animals were weighed and samples were procured for the determination of radioactivity and microscopic evaluation. A portion of the liver was taken from all animals killed on day 21 and microsomes were prepared in order to determine the localization of radioactivity in this fraction. DNA, RNA and protein assays were conducted on the liver homogenates. RNA, protein, cholesterol and phospholipid levels were established from these homogenates (Norback & Allen, 1972). In addition, enzymatic activities of aromatic hydroxylase, nitroreductase, esterase, glucose-6-phosphatase and *N*-demethylase were assayed using substrate concentrations of 100 mM-aniline, 3 mM-*p*-nitrobenzoic acid, 3·5 mM-*p*-nitrophenyl acetate, 40 mM-glucose-6-phosphate and 6 mM-aminopyrine, respectively, as previously reported (Norback & Allen, 1972). The activity of glu-

cose-6-phosphatase in the homogenate was also established, in order to determine the percentage of microsomes recovered. For the determination of radioactivity in the tissues, excreta and liver microsomes, aliquots were solubilized in Unisol and Unisol-Complement (Isolab) or oxidized in a Packard Tri-Carb sample oxidizer as previously described (Van Miller, Hsu & Allen, 1975).

The tissue samples selected for light-microscopic evaluation were placed in 10% neutral buffered formalin for 24 hr, dehydrated, embedded in paraffin and sectioned. The sections, 5 μm thick, were stained with haematoxylin and eosin and evaluated histologically. Small segments of the liver were also prepared for electron microscopy by being fixed in osmium tetroxide buffered with veronal acetate for 1·5 hr (Caulfield, 1957) and subsequently embedded in an epoxy-resin mixture (Mollenhauer, 1964). Sections of the tissue were cut on an ultratome, placed on uncoated copper grids, stained with uranyl acetate and examined with an RCA-EMU-3G electron microscope.

RESULTS

During the initial 14 days, the animals ate well but showed a weight loss that became more severe as the experiment progressed. At no time were the animals anorectic. Invariably the stomach and intestinal tract of the animals that died or were killed contained abundant ingesta. In addition, all of the TCDD-treated animals showed an excessive loss of hair, particularly during the latter part of the experiment. By day 25, half of the animals that had not been involved in the sequential kills were dead. The surviving animals showed an average weight loss of 76 g during the course of the experiment. The haematological evaluations conducted on the blood of rats killed on day 21 showed no appreciable changes in their haemoglobin levels, haematocrits or white-cell counts. Total serum lipids, cholesterol, triglycerides and protein, as well as the albumin/globulin ratio, were within the normal range.

Within the first 72 hr after the administration of [^{14}C]TCDD, over 25% of the radioactivity had been eliminated by the alimentary route. During the subsequent 18 days, between 1 and 2% was excreted daily. By day 21, 52·3 ± 6·3% (± SD) of the radioactivity had been eliminated with the faeces. The radioactivity of the urine during the initial 12 days ranged daily between 0·10 and 0·20% of the total dose; thereafter, the percentage increased from 0·25 to 0·43% by day 21. The total percentage excreted in the urine was 4·54 ± 0·7%.

It was apparent at autopsy that there was an increase in relative liver weight (Table 1) which was particularly significant in that the absolute weights of the livers from the treated animals increased while body weight diminished. Microscopically the liver cells were seen to be enlarged and to contain numerous lipid droplets. Ultrastructurally the cytoplasm of the hepatic cells showed a decided proliferation of the smooth endoplasmic reticulum (ER) and the presence of concentric membrane arrays (Figs 1 & 2). The latter membranes were continuous with the rough and smooth ER and frequently encircled lipid droplets, mitochondria and other organelles. The thymic atrophy which was observable grossly at day 1 was characterized by a paucity of cortical thymocytes at this time and throughout the experiment (Figs 3 & 4).

Table 1. *Hepatic changes found in rats 21 days after intubation with 50 μg [^{14}C]TCDD/kg body weight*

Analysis		Values for Control group	Values for Test group
Liver weight (g/100 g body weight)		2·76 ± 0·07	4·47 ± 0·44*
Homogenate:	DNA (mg/g liver)	3·32 ± 0·19	2·66 ± 0·33*
	RNA (mg/mg DNA)	2·72 ± 0·10	2·88 ± 0·25
	protein (mg/mg DNA)	82·9 ± 2·3	88·4 ± 15·2
Microsomal:	protein (mg g liver)	20·5 ± 1·6	8·34 ± 0·86*
	RNA (μg mg protein)	151 ± 6	164 ± 45
Phospholipid (μg mg protein)		439 ± 54	576 ± 34*
Cholesterol (μg/mg protein)		15·6 ± 4·3	27·5 ± 4·2*
N-Demethylase (nmol formaldehyde/30 min)†		97·8 ± 5·8	143 ± 39
Aromatic hydroxylase (nmol *p*-aminophenol/30 min)†		26·2 ± 2·6	12·4 ± 3·9*
Nitroreductase (nmol *p*-aminobenzoate/hr)†		9·88 ± 2·22	10·9 ± 2·6
Glucose-6-phosphatase (μmol PO_4/15 min)†		7·49 ± 0·57	3·70 ± 0·58*
Esterase (μmol *p*-nitrophenol/min)†		7·84 ± 2·05	3·70 ± 1·17*

†Per mg microsomal protein.

Values are means ± 1 SD for groups of five rats; those marked with an asterisk differ significantly from the control value: *$P < 0·01$.

When the rats were killed, it was demonstrated that the highest radioactivity was located in the liver (Table 2), the levels per gram of tissue being 6–7 times greater in this organ than in other tissues. The level of radioactivity in the liver was fairly constant during the initial 14 days, but during the subsequent 7 days it decreased by some 50%. When fractions of these livers were evaluated, the radioactivity was found to predominate in the microsomal fraction (Table 3). After 24 hr the adipose tissue was second to the liver in the level of radioactivity (Table 2), but this level represented only a small fraction of that present in the liver. Tissues not mentioned in Table 2 contained less than 0·1% of the total radioactivity per gram of tissue. When the various tissues were evaluated in relation to their total radioactivity, the level in the liver far exceeded that present in any other tissue (Table 4). Although the radioactivity in the skin was relatively low when expressed on a 'per gram' basis, it was second only to the liver when the total weight of the skin was considered. The stomach and intestinal tract initially contained relatively high levels of radioactivity but these decreased rapidly during the succeeding days (Table 2). When the levels in the various tissues and excreta were considered, it was possible to account for over 90% of the radioactivity administered as [^{14}C]TCDD.

Biochemically the liver homogenates prepared from the TCDD-treated rats at day 21 showed no appreciable change in the concentration of protein and RNA (Table 1). There was, however, a significant decrease in the level of DNA/g liver. The microsomes from these homogenates had less protein and more phospholipid and cholesterol. In addition, there was a significant reduction in the activity of esterase, glucose-6-phosphatase and aromatic hydroxylase.

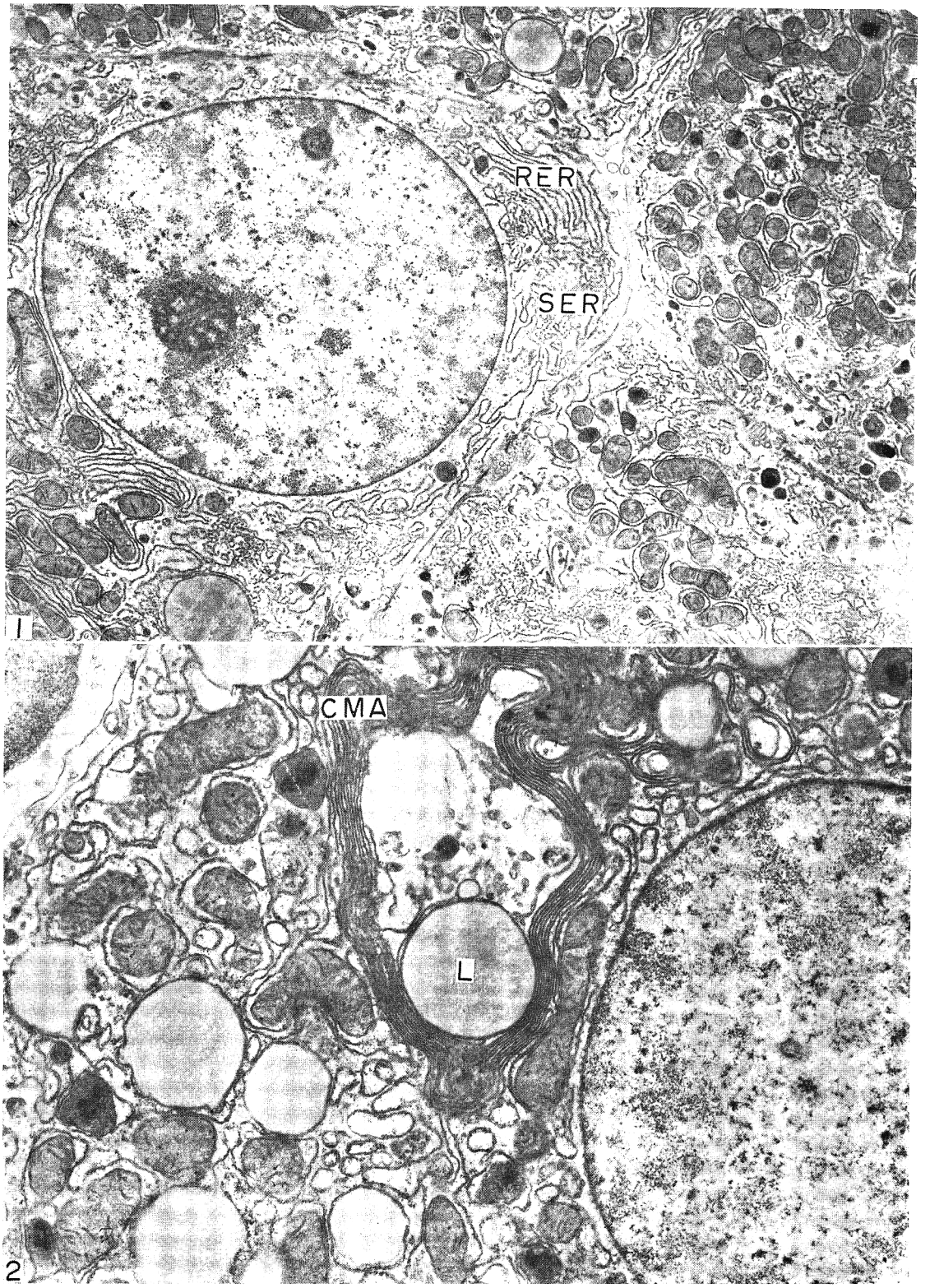

Fig. 1. Liver section from a control rat, showing abundant long segments of rough endoplasmic reticulum (RER) that have assumed a lamellar profile. The smooth endoplasmic reticulum (SER) is arranged in packets at the periphery of the RER. Uranyl acetate × 8190.

Fig. 2. Liver section from a TCDD-intubated rat showing the rough endoplasmic reticulum (RER) in a more serpentine arrangement and much more vesiculated than in the control liver. Segments of RER are continuous with the large concentric membrane arrays (CMA). A large lipid droplet (L) is encircled by these membranes. Uranyl acetate × 15,330.

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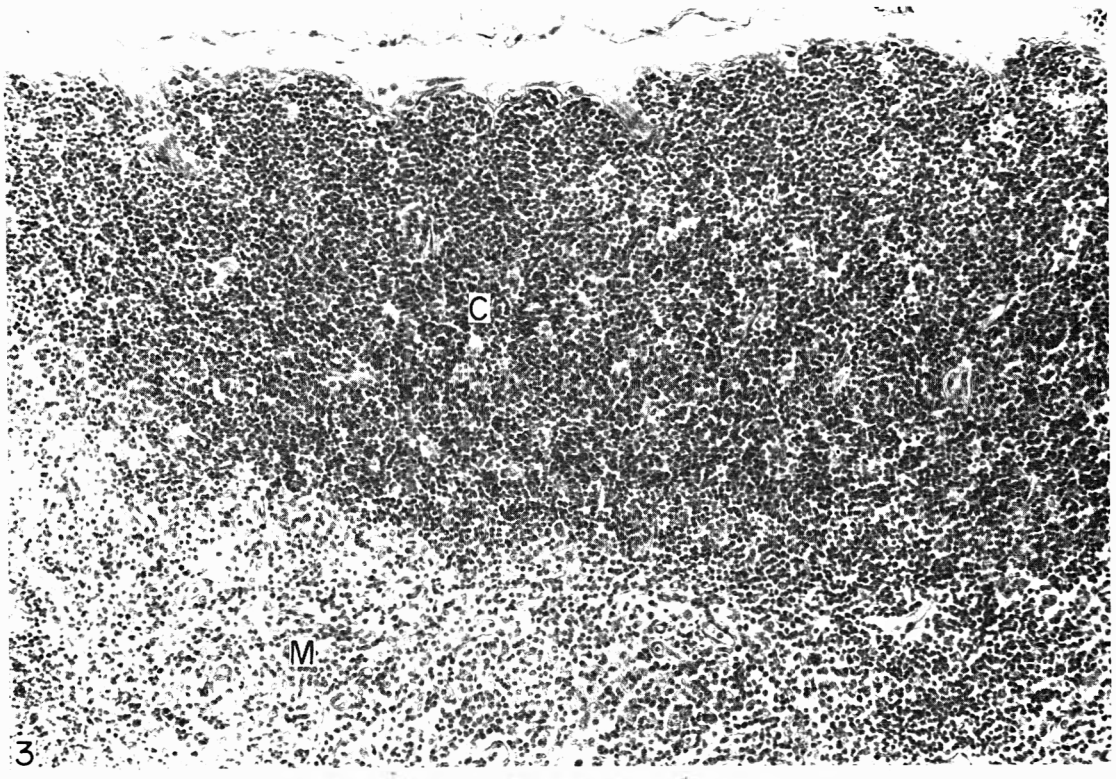

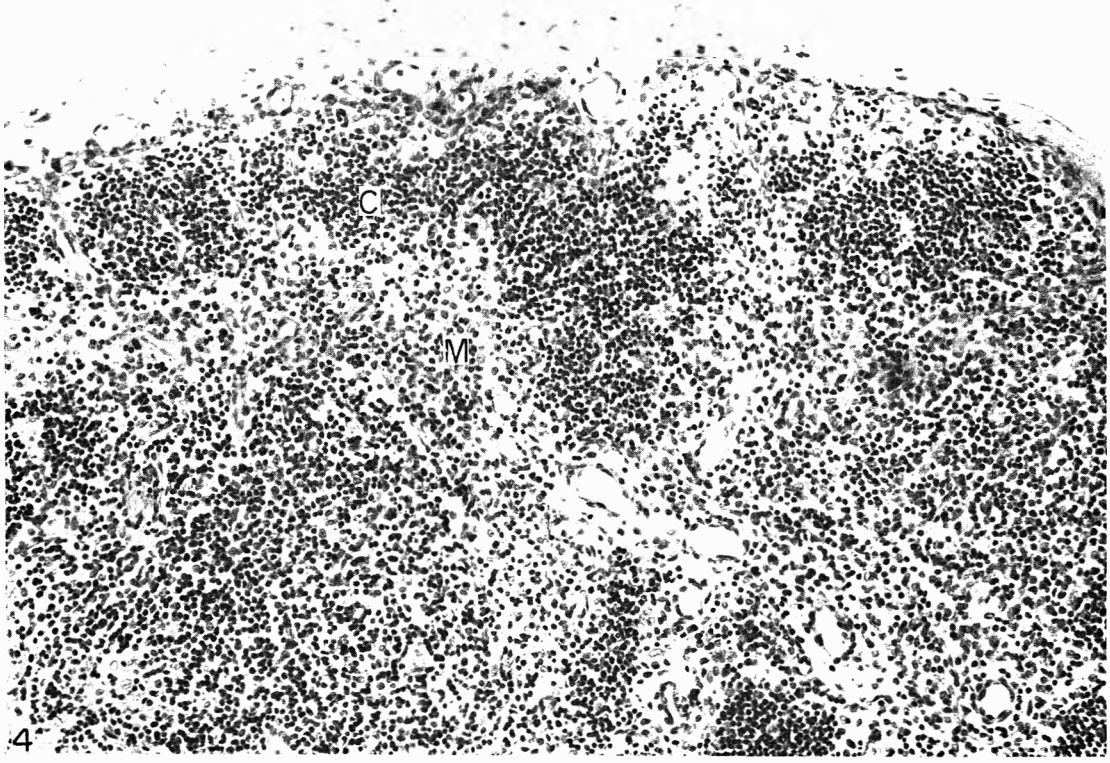

Fig. 3. Section of the thymus from a control rat showing a wide zone of cortical thymocytes (C) and a distinct medullary region (M). Haematoxylin and eosin ×100.

Fig. 4. Section of a TCDD-treated thymus characterized by a lack of any sharp line of demarcation between the cortex (C) and medulla (M). Haematoxylin and eosin ×100.

Table 2. *Distribution of ^{14}C activity in the tissues of rats 1–21 days after administration of a single dose of 50 μg [^{14}C]TCDD/kg*

Tissue	^{14}C activity* (% of dose/g tissue) at day					
	1	3	5	7	14	21
Liver	8·39 ± 0·81	8·55 ± 1·65	7·20 ± 1·10	6·27 ± 0·61	6·04 ± 2·79	3·16 ± 0·60
Adrenal	0·34 ± 0·14	0·51 ± 0·13	0·38 ± 0·13	0·33 ± 0·08	0·22 ± 0·11	0·38 ± 0·16
Stomach and small intestine	0·25 ± 0·08	0·16 ± 0·12	0·08 ± 0·02	0·08 ± 0·02	0·08 ± 0·03	0·07 ± 0·02
Large intestine	1·14 ± 0·42	0·34 ± 0·04	0·25 ± 0·07	0·25 ± 0·04	0·17 ± 0·10	0·18 ± 0·06
Adipose tissue	0·77 ± 0·29	1·26 ± 0·51	0·99 ± 0·30	0·98 ± 0·28	0·94 ± 0·44	0·97 ± 0·45
Skin	0·25 ± 0·07	0·20 ± 0·05	0·16 ± 0·06	0·14 ± 0·03	0·08 ± 0·04	0·12 ± 0·05

*Total recovery of [^{14}C]TCDD in tissues and excreta exceeded 90% of administered dose.
Values are means ± 1 SD for groups of five rats.

Table 3. *Localization of ^{14}C in the liver microsomes of rats given a single dose of [^{14}C]TCDD*

Days after treatment	Relative glucose-6-phosphatase activity (microsomes/homogenate)	Percentage of liver radioactivity in microsomes (% in microsome/G-6-Pase activity)*
5	0·306 ± 0·045	93·89 ± 4·77
7	0·357 ± 0·035	90·43 ± 4·42
14	0·287 ± 0·030	95·95 ± 5·96
21	0·334 ± 0·051	82·75 ± 12·60

*Calculation assumes that enzymatic activity is limited exclusively to the hepatic microsomal fraction.
Values are means ± 1 SD for groups of five animals.

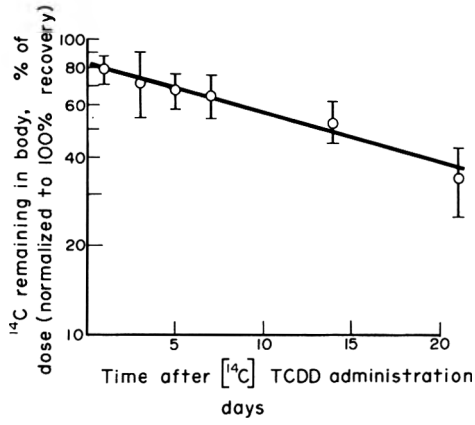

Fig. 5. Rate of clearance of ^{14}C from the tissues of rats given [^{14}C]TCDD by gastric intubation. Each point represents the mean ± SD for groups of five rats.

The biological half-life of the [^{14}C]TCDD was determined as 16·3 ± 2·3 days when the total radioactivity in the entire body was plotted against time (Fig. 5). However, when the amount of radioactivity in the daily excreta was subtracted from the total dose and the log of these values was plotted against time, the half-life of the ^{14}C was 21·3 ± 2·9 days. The half-life value was not altered appreciably when the radioactivity in the faeces during the initial 48 hr was excluded from this calculation, because it probably represented unabsorbed TCDD.

DISCUSSION

If one assumes that a large percentage of the ^{14}C activity excreted in the faeces during the initial 72 hr represents unabsorbed TCDD, over 75% of the administered dose was absorbed from the gastro-intestinal tract. This would tend to be substantiated by the data that show a decrease from over 18% to only 2·3% of the activity during the initial 3 days. In addition, the ^{14}C activity in the large intestine declined rapidly after day 1. Of that which was absorbed from the gastro-intestinal tract, approximately 75% was deposited in the liver. Another tissue having considerable activity was the adipose tissue. However, in this experiment, the adipose tissue was not a major site

Table 4. *Tissues with the largest percentage of ^{14}C activity 1–21 days after administration of [^{14}C]TCDD*

Tissue	^{14}C activity (% of total ^{14}C activity in dose) at day					
	1	3	5	7	14	21
Liver	55·55 ± 5·01	54·21 ± 14·32	54·22 ± 6·40	53·66 ± 8·03	45·05 ± 5·14	23·86 ± 4·14
Skin*	9·43 ± 2·9	6·63 ± 1·44	5·88 ± 2·60	5·32 ± 1·24	2·71 ± 1·27	3·8 ± 1·6
Large intestine†	4·6 ± 1·4	1·39 ± 0·37	0·90 ± 0·30	0·93 ± 0·17	0·74 ± 0·60	0·66 ± 0·25
Stomach and small intestine†	1·81 ± 0·56	1·09 ± 0·79	0·49 ± 0·10	0·58 ± 0·16	0·53 ± 0·13	0·44 ± 0·12

*Animals were pelted at autopsy to facilitate total evaluation of localization.
†Contents were removed and gut was weighed before evaluation.
Values are means ± 1 SD for groups of five rats.

of localization of TCDD because of the cachectic state of the animals.

It is of interest that the levels of radioactivity in the urine increased as the experiment progressed. These findings are in contrast to the observation of Piper, Rose & Gehring (1973), who reported only minute amounts of radioactivity in the urine after day 8. Vinopal & Cassida (1973) detected only unmetabolized TCDD in the liver and faeces of mice, but made no mention of the urine. In the present study the radioactivity in the urine was highest from wk 2 to 3 and may have represented metabolites of TCDD. Research is now in progress to clarify the nature of this radioactive material in the urine.

The two values for the biological half-life ($16{\cdot}3 \pm 2{\cdot}3$ and $21{\cdot}3 \pm 2{\cdot}9$ days) obtained first by measuring the decrease in radioactivity in the tissues with time and secondly by calculating the remaining body burden from excreted radioactivity are probably not significantly different. In fact, these data agree quite well with the value of $17{\cdot}4 \pm 5{\cdot}6$ days reported by Piper *et al.* (1973).

Of particular interest was the affinity of the liver and more specifically the microsomal fraction for the radioactivity of [^{14}C]TCDD. In 1969, Norback and Allen postulated that the proliferated endoplasmic reticulum was a likely site for the localization of dioxin. The currently reported data, as well as those of Vinopal & Casida (1973), tend to support this postulation. Subsequently, Norback & Allen (1972) reported on the compositional alteration of the lipid portion of the hepatic microsomes of rats fed chlorinated aromatic hydrocarbons. It was proposed that the phospholipid and cholesterol levels of the new membranes would be compatible with an altered structure for the localization of foreign lipid-soluble substances. The study now reported tends to support the above, in that over 90% of the radioactivity of [^{14}C]TCDD in the liver was located in the compositionally altered microsomal fraction.

According to the data presented by Lucier, McDaniel, Hook, Fowler, Sonawane & Faeder (1973), TCDD causes an increase in benzopyrene hydroxylase, aromatic hydroxylase, glucuronyl transferase, cytochrome *P*-450 and cytochrome b_5 when given in a single dose of 0·2–0·5 μg/kg. This increased activity persisted at a high level for 16 days and then declined rapidly. In the study reported here, there was a reduction in the activities of esterase, glucose-6-phosphatase and aromatic hydroxylase. It is known that the hepatic endoplasmic reticulum undergoes hyperplasia, which is associated with hyperactivity of microsomal enzymes. These membranes may remain hyperplastic but eventually become hypoactive enzymatically (Hutterer, Schaffner, Klion & Popper, 1968).

The decided proliferation of the hepatic endoplasmic reticulum observed electron-microscopically in the present study would suggest, under most circumstances, an increase in enzymatic activity. However, in this particular case the enzymatic activity of these membranes was less than that of the controls. Thus it appears that these hyperplastic membranes had progressed from a hyperactive to a hypoactive state.

As occurs with many other chlorinated aromatic hydrocarbons, a considerable portion of ingested dioxin became localized in the adipose tissue. However, there seems to be a difference in the turnover rate between the dioxins and other chlorinated aromatic hydrocarbons, such as the polychlorinated biphenyls (PCBs), which also are deposited in large amounts in the adipose tissue. When an animal fed PCBs is deprived of food either through illness or restricted intake, the fat along with the PCBs is readily metabolized and transferred to the liver. This is associated with a decrease in PCB concentration in the adipose tissue and an increase in liver PCB (Allen, Carstens & Barsotti, 1974). With the dioxins there are indications that this is not the case. All the animals lost weight following TCDD treatment and there was a decided depletion of depot fat. However, the level of radioactivity in the adipose tissue remained constant, while that of the liver showed a decided decrease.

Thymic hypoplasia is a constant feature of TCDD intoxication in most animal species. Vos *et al.* (1973) reported thymic atrophy and lymphocytopenia in guinea-pigs and mice, while rats showed only thymic regression. Such was also the case in the study now reported, in which only a small zone of the cortical thymus was apparent, yet the spleen and lymph nodes appeared unaffected. There also seemed to be no effect on the myelopoietic tissue, since the bone marrow was normal and the haemoglobin and haematocrits were unaffected. Vos *et al.* (1973) have also observed suppression and humoral and cell-mediated immunity in guinea-pigs and mice, while rats were unaffected. Although the evaluation of the immunological competence of the TCDD rats was not included in this experiment, it can be said that at the dose level used there were no alterations in the serum-globulin fraction of these rats.

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LACK OF DOMINANT LETHALITY IN RATS TREATED WITH POLYCHLORINATED BIPHENYLS (AROCLORS 1242 AND 1254)*

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Abstract—The possible mutagenicity of the polychlorinated biphenyls (PCBs) was investigated using the dominant lethal test. Aroclor 1242 was given to male Osborne–Mendel rats by oral intubation in a single dose of 625, 1250 or 2500 mg/kg, or in five daily doses of 125 or 250 mg/kg. Aroclor 1254 was given by oral intubation in five daily doses of 75, 150 or 300 mg/kg. The rats were then mated with untreated females for the following 10–11 wk. An additional group of male rats given five daily doses of Aroclor 1254 at 150 mg/kg was starved overnight before the admittance of females. In a feeding study, males were given Aroclor 1254 at a dietary level of 25 or 100 ppm for 70 days and then were mated with untreated females for 1 wk. Triethylenemelamine (TEM) was used as the positive control compound. Results, except for those obtained with TEM, showed only random, unrepeatable effects that were not related to dose or to the stage of spermatogenesis.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are industrial chemicals which have gained worldwide recognition as contaminants of ecosystems. During the past few years there has been an increasing amount of interest in the biological effects of PCBs in man and experimental animals.

In the 'Yusho' incident in Japan, approximately 1000 people consumed rice oil contaminated with PCBs (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972). The symptoms described were dark-brown pigmentation of the nails, acne-like skin eruptions, pigmentation of the skin, increased eye discharge, hyperaemia of the conjunctiva and swelling of the upper eyelids.

The PCBs also exert a variety of effects in experimental animals. Studies have shown that they affect steroid balance in boars (Platonow, Liptrap & Geissinger, 1972), produce hyperplasia and dysplasia of the gastric mucosa in primates (Allen & Norback, 1973) and synergize the toxicity of pesticide residues in human cells in culture (Lichtenstein, Schulz, Fuhremann & Liang, 1969). Kimbrough (1971) reported that PCBs caused reproductive effects in rats; fewer and smaller pups were born and their survival time was decreased. Keplinger, Fancher & Calandra (1971) also noted poor hatchability of eggs, decreased thickness of egg shells and loss of body weight in chickens fed PCBs.

Very little information is available about the possible mutagenicity of the PCBs. Studies were begun, therefore, at the Food and Drug Administration (FDA) in an attempt to assess their mutagenicity as determined by cytogenetics and dominant lethality. The cytogenetic effects of Aroclors® 1242 and 1254 in rats have been investigated (Green, Carr, Palmer & Oswald, 1975) and this report presents the results of dominant lethal studies of Aroclors 1242 and 1254 in rats after acute or multiple (5-day) oral administrations and of Aroclor 1254 after 70 days of dietary feeding.

EXPERIMENTAL

Materials. Aroclor 1242 was obtained from the Monsanto Chemical Co., St. Louis, Mo., and Aroclor 1254 from a stock supply maintained by the Chemical Technology Branch of the FDA. According to information supplied by Monsanto, all samples were free of contamination by the dibenzofurans. A known mutagen, triethylenemelamine (TEM), served as the positive control compound in the acute and multiple-dose studies, and was obtained from the Lederle Laboratory Division of American Cyanamid Corp., Pearl River, N.Y.

Animals and dosing. Random-bred male and female Osborne–Mendel rats, weighing 275–350 and 200–250 g, respectively, were used for the acute and multiple-dose studies. Ten males were randomly assigned to each group except the control group in the acute study of Aroclor 1242, which consisted of 15 males. In these studies, the Aroclors were administered as solutions in corn oil, dilutions being such that all animals received 1 ml/kg body weight. Controls received a similar amount of corn oil. In the feeding study, Osborne–Mendel male rats weighing 250–300 g were used and Aroclor 1254 was added directly to the food. TEM was dissolved in water and administered orally or ip.

Acute and multiple-dose studies. In the first study,

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Table 1. *Toxicity of Aroclors 1242 and 1254 in Osborne–Mendel rats after administration by oral intubation or in the diet*

Treatment	Dose (mg/kg/day)	Duration of treatment (days)	No. dead/no. treated	Mean weight loss (g)
Control	Corn Oil	1	0/15	—
Aroclor 1242	625	1	0/10	—
	1250	1	1/10	5
	2500	1	2/10	20
	125	5	1/10	—
	250	5	0/10	21
Control	Corn Oil	5	0/10	—
Aroclor 1254	75	5	0/10	7
	150	5	0/10	23
	300	5	5/10	56
Control	Lab. Chow	70	2/26	—
Aroclor 1254	25*	70	3/27	—
	100*	70	2/28	24

*Dietary concn in ppm.

Aroclor 1242 was given to male rats by oral intubation in single doses of 625, 1250 or 2500 mg/kg. In the second study, male rats were given Aroclor 1254 for 5 days in doses of 75, 150 or 300 mg/kg/day. Each male, after treatment, was mated with two females weekly for the following 10 wk. As part of the second study, males in an additional group given 150 mg/kg/day were starved overnight after the last treatment prior to the admittance of females. This procedure was followed after a suggestion that lipolysis during starvation may augment the levels of circulating PCBs and thereby increase the possible dominant lethality.

In the third study, males were given five daily doses of Aroclor 1242 at 125 or 250 mg/kg/day or of Aroclor 1254 at 75 or 150 mg/kg/day. This third study was performed not only to repeat part of the second study but also to assess whether a significant reduction in the number of dividing spermatogonial cells, which had been found to occur in rats treated with Aroclor 1242 (Green *et al.* 1975), could influence the reproductive performance of males. Male animals were therefore mated one additional week (a total of 11 wk) to ensure 3 wk of spermatogonial examination.

The females were killed in mid-pregnancy and examined for the numbers of corpora lutea, the numbers of living implantations and the numbers of implantations regarded as early and late deaths. The categories of early and late deaths were combined under the heading 'dead implantations'. Pre-implantation loss was determined directly by subtracting the number of implantations from the number of corpora lutea.

70-Day feeding study. Test groups of 27 and 28 male rats were fed Purina Laboratory Chow with Aroclor 1254 added at levels of 25 and 100 ppm, respectively, while a control group of 26 rats was fed the untreated chow. After 70 days, ten rats were randomly selected from each group, the remaining rats being allocated for studies of the biochemical effects of Aroclor 1254 and its influence on the metabolism of aflatoxin B_1, work which will be reported elsewhere. Each of the ten selected rats was then housed with two females for 1 wk to provide information about dominant lethality, as outlined in the acute and multiple-dose studies.

Statistical evaluation of results. The average number of implantations per pregnant female and the average number of corpora lutea per pregnant female were analysed for statistical significance by the *t* test. For the average number of dead implantations per pregnant female, the average number of pre-implantation losses and the proportion of dead implantations to total implantations, data were transformed by the Freeman–Tukey arc-sine procedure (Mosteller & Youtz, 1961) before analysis by the *t* test. The proportions of females with one or more and with two or more dead implantations were analysed by the chi-square test.

RESULTS

Toxicity

Table 1 shows the toxicity of Aroclors 1242 and 1254 at the doses utilized in this investigation. The intent was to achieve a toxic condition at the uppermost levels and then to utilize lower 'non-toxic' doses to assess dominant lethality. The Aroclor 1254 dose of 300 mg/kg given for 5 days caused considerable weight losses and deaths. At other dose levels weight losses and the numbers of deaths were minimal.

Table 2. *Mean numbers of implantations and dead implantations per pregnant female after treatment of males with a single orally intubated dose of Aroclor 1242*

Wk no.	Implantations/pregnant female after treatment of males with: Vehicle control† P	I	DI	Aroclor 1242 in a dose (mg/kg) of: 625 P	I	DI	1250 P	I	DI	2500 P	I	DI	TEM (0·5 mg/kg, ip) P	I	DI
1	21	12·7	0·90	13	12·2	0·54	3	13·0	0·33	10	11·5	0·20	13	13·7	1·54
2	20	11·2	0·70	19	10·8	0·47	8	12·6	0·88	12	10·6	0·83	13	11·1	3·08**
3	23	12·1	0·87	16	10·6	0·69	6	11·7	0·67	15	12·1	0·87	11	12·5	2·36*
4	21	11·9	0·76	19	12·8	0·47	8	11·4	0·38	12	13·1	0·58	13	11·8	5·85**
5	19	11·6	0·53	16	11·6	0·50	9	12·8	0·89	13	11·7	0·69	14	11·5	0·86
6	23	12·5	0·78	18	11·9	0·72	9	11·8	0·22	15	12·5	0·93	13	12·1	0·38
7	24	11·8	1·08	17	11·5	0·76	9	13·2	0·67	15	12·0	0·93	16	11·7	0·50
8	24	12·2	0·96	19	12·4	0·53	9	12·4	0·22	15	13·1	0·60	15	12·2	0·67
9	24	10·7	0·75	17	12·9	0·47	8	13·2	0·62	13	11·8	0·38	12	12·8	0·75
10	26	11·7	1·19	19	12·3	0·89	9	12·2	0·33	15	12·7	0·93	15	12·7	1·13

P = No. of pregnancies I = No. of implantations/pregnancy DI = No. of dead implantations/pregnancy
TEM = Triethylenemelamine

†Corn oil (1 ml/kg).
Values marked with asterisks differ significantly from the control value: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Table 3. *Mean numbers of implantations and dead implantations per pregnant female after treatment of males for 5 days with Aroclor 1254 by oral intubation*

Wk no.	Implantations/pregnant female after treatment of males with: Vehicle control† P	I	DI	Aroclor 1254 in doses (mg/kg/day) of: 75 P	I	DI	150 P	I	DI	150‡ P	I	DI	300 P	I	DI	TEM (0·2 mg/kg/day, ip) P	I	DI
1	11	10·9	0·18	13	11·7	0·62	13	11·2	1·15*	14	10·9	0·50	5	12·6	0·20	16	11·2	2·31**
2	15	11·4	1·13	20	11·9	1·20	13	10·5	0·85	14	11·3	1·00	7	12·4	1·43	17	10·8	3·29**
3	17	11·4	1·29	16	11·9	1·06	15	12·3	0·53	19	10·7	0·63	8	11·6	0·62	16	11·3	3·06**
4	13	11·0	0·85	16	11·9	1·12	17	12·2	1·06	18	11·6	0·83	8	10·9	1·12	18	10·1	4·11**
5	16	12·0	0·94	20	11·8	1·70*	19	11·7	1·26	17	10·8	0·94	13	10·5	0·30	18	11·6	2·39*
6	15	11·0	0·93	19	11·6	1·42	16	11·9	1·19	17	12·0	1·06	9	11·0	0·22	18	11·2	0·83
7	16	11·0	0·56	19	11·9	0·95	18	11·6	0·56	18	11·4	0·22	8	10·2	0·25	15	10·9	0·20
8	15	11·6	1·07	19	11·0	0·95	18	11·7	0·50	17	11·3	0·88	9	11·1	2·67*	17	10·9	0·53
9	18	11·1	1·06	15	10·9	1·40	16	10·8	0·75	16	11·3	1·31	8	10·5	1·38	17	11·1	0·24
10	16	11·8	1·12	17	11·8	1·35	20	11·6	0·95	17	9·7*	1·00	9	10·9	1·11	16	11·8	1·19

P = No. of pregnancies I = No. of implantations/pregnancy DI = No. of dead implantations/pregnancy
TEM = Triethylenemelamine

†Corn oil (1 ml/kg).
‡Males were starved overnight before the admittance of females.
Values marked with asterisks differ significantly from the control value: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Acute and multiple-dose studies

As seen in Table 2 females mated with males given the acute treatment with Aroclor 1242 were unaffected with respect to the number of implantations and the number of dead implantations per pregnant female. TEM produced statistically significant post-implantation losses in wk 2, 3 and 4 (Table 2).

Similar results were obtained in the multiple-dose studies of Aroclor 1254. In wk 10, the number of implantations was significantly decreased in females mated with males that were starved after the last dose (Table 3), but no biological significance was attached to this result. Table 3 shows that a statistically significant increase in dead implantations (post-implantation loss) occurred at wk 1 in females mated with males given five daily doses of Aroclor 1254 at 150 mg/kg. Significant increases were also obtained for wk 5 and 8 at the dose levels of 75 and 300 mg/kg, respectively. These responses were also considered random increases and presumed to be of no biological significance. TEM produced significant increases in dead implantations in wk 1–5.

In the repeat experiment with Aroclor 1254 given in five daily doses of 75 and 150 mg/kg, significant decreases in implantations were noted in wk 3 and 5 (Table 4). At wk 11, a statistically significant post-implantation loss was observed in females mated with males treated with Aroclor 1242 at 250 mg/kg for 5 days (Table 4). These responses were considered to be similar to the previous single-week responses. TEM produced significant increases in post-implantation loss at wk 4, 5 and 11.

There was no evidence from this study, in which spermatogonia were sampled for 3 wk (wk 9, 10 and 11), that a reduction in the number of dividing spermatogonial cells impaired the reproductive performance of male animals. This conclusion was based on the finding that the numbers of implantations per pregnant female were normal in these groups.

70-Day feeding study

There were no significant differences between the treated and control groups in the numbers of implantations and dead implantations per pregnant

Table 4. *Mean numbers of implantations and dead implantations per pregnant female after treatment of males for 5 days with Aroclors 1242 or 1254 by oral intubation*

Wk no.	Implantations/pregnant female after treatment of males with: Vehicle control† P	I	DI	Aroclor 1242 in doses (mg/kg/day) of: 25 P	I	DI	250 P	I	DI	Aroclor 1254 in doses (mg/kg/day) of: 75 P	I	DI	150 P	I	DI	TEM (0·2 mg/kg/day, ip) P	I	DI
1	14	11·4	0·50	5	12·4	0·60	6	12·0	0·17	10	12·1	0·40	13	12·8	0·54	14	14·6	0·86
2	12	11·1	0·92	12	10·8	0·42	9	9·0	0·22	14	10·0	1·29	8	7·6	0·25	12	11·2	1·58
3	16	12·1	1·62	15	11·5	1·20	17	11·7	0·47	12	8·9*	1·00	14	9·4*	0·71	17	11·1	0·76
4	16	10·2	0·88	16	11·8	1·12	18	11·5	1·06	14	10·1	1·14	12	12·8	0·67	18	11·6	1·78*
5	13	12·4	0·69	16	12·2	0·25	19	12·3	0·42	16	7·2**	0·31	15	12·2	0·73	17	11·0	1·76*
6	16	11·3	0·38	15	12·5	0·53	18	12·9	0·44	17	11·3	1·12	14	11·9	0·21	18	13·2	0·83
7	15	10·9	0·33	13	12·4	0·23	20	10·8	0·65	17	12·1	0·53	13	11·6	0·08	18	15·7	0·50
8	14	11·0	0·36	16	10·1	0·25	18	11·7	0·78	17	10·4	0·94	17	9·6	0·71	18	11·6	0·39
9	15	9·9	0·53	16	10·0	0·38	19	10·3	0·74	16	10·6	0·31	15	10·9	0·27	18	10·7	0·56
10	16	10·2	0·31	16	10·1	0·25	18	9·3	0·61	16	10·2	0·69	16	10·1	1·44	16	10·3	0·38
11	12	10·7	0·17	16	10·8	0·38	15	11·1	1·13**	16	12·2	0·38	16	11·4	0·31	19	10·0	0·63*

P = No. of pregnancies I = No. of implantations/pregnancy DI = No. of dead implantations/pregnancy
TEM = Triethylenemelamine

†Corn oil (1 ml/kg).
Values marked with asterisks differ significantly from the control values: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

female in the feeding study. The mean values for the 16, 15 and 14 pregnancies resulting from matings with males fed, respectively, on diets containing 0 (control), 25 or 100 ppm Aroclor 1254 were 13·4, 12·8 and 12·8 for implantations and 0·31, 1·00 and 0·29 for dead implantations.

DISCUSSION

Acute and multiple-dose studies

The most reliable indication of a dominant lethal effect is a statistically significant increase in the number of dead implantations per pregnant female (Epstein, Arnold, Andrea, Bass & Bishop, 1972; Green & Springer, 1973). A reduction in the number of implantations per pregnant female may or may not be due to dominant lethality for reasons previously outlined (Green, Palmer & Legator, 1972). The remaining indexes investigated but excluded as tabular information are ancillary to determining the degree of the dominant lethal effect and clarifying possible ambiguous results. Inasmuch as the PCBs produced no consistent dominant lethal effects, tabular information was restricted to the number of dead implantations and the number of implantations per pregnant female.

Two factors were considered in concluding that the effects produced by the PCBs were random increases. The first was that, in contrast to our results with the PCBs, dominant lethal effects are rarely restricted to 1 wk. The responses usually correlate with specific spermatogenic stages, as exemplified by the results obtained with the positive control compound TEM shown in Tables 2, 3 and 4. Secondly, we were unable to repeat the significant effects that were obtained. A comparison of the significant data in Table 3 with those in Table 4 illustrates this point.

70-Day feeding study

The feeding study conducted in this investigation covered the spermatogenic cycle of the rat. It was obvious from the results obtained that the PCBs possessed no dominant lethal potential at the levels tested. This study served another purpose, however. One of the problems in evaluating dominant lethal results emanates from the massive amount of data generated (Green & Springer, 1973). One method of decreasing the amount of information would be to treat male animals over the entire spermatogenic cycle and mate only once. This method obscures the specific spermatogenic stage that is affected, but would lead to significant effects in the first week of mating. Additional information about positive control compounds would aid in the development of this approach to dominant lethal testing.

This investigation, taken collectively, demonstrates that Aroclors 1242 and 1254 are not mutagenic as defined by the induction of dominant lethality.

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GENERATION AND INHALATION TOXICITY OF DICHLOROACETYLENE

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Abstract—An apparatus is described for the generation and analytical control of constant dichloroacetylene (DCA) concentrations over the range of 10 to several thousand ppm throughout an arbitrary period of time, for the exposure of experimental animals. Trichloroethylene vapours are drawn in a stream of nitrogen at 120°C across dried KOH. The DCA/N_2 mixture is monitored by infra-red spectrography, the infra-red absorption being standardized by combustion of the gas mixture in the nitrogen stream and by the subsequent determination of the chloride obtained, using a standard curve. The formation of phosgene as a degradation product in the presence of oxygen can be determined by infra-red spectrography or photometry.

From tests on mice, the LC_{50}s of DCA for a 1-hr and 6-hr inhalation were calculated to be 124 (108–153) and 19 (16–22·6) ppm, respectively. The lungs and livers of exposed mice showed no abnormalities, pathological changes being limited to extensive necrosis in the kidneys. The renal lesions, which mainly affected the proximal tubules, were the probable cause of death. In addition, degenerative lesions found in certain nuclear regions of the brain suggested some parallel with the neurotoxic symptoms observed in man.

INTRODUCTION

Exposure to trichloroethylene (Tri) is occasionally associated with the occurrence of an unusual but highly characteristic picture of intoxication, associated with irreversible lesions of the sensitive cranial nerves, particularly the trigeminal nerve. Numerous reports have appeared describing this acute syndrome, which differs completely from that found in typical Tri intoxication. According to Humphrey & McClelland (1944), A. E. Cox was the first investigator to incriminate dichloroacetylene (DCA), a breakdown product of Tri, as the actual toxic agent, but analytical proof of this effect is, as yet, lacking. We have described two cases of intoxication involving not Tri but an intermediate in the production of polyvinylidene chloride copolymers (Henschler, Broser & Hopf, 1970). The technical product was shown to contain several chlorinated aliphatic C_2 hydrocarbons which could have led to the formation of di- and monochloroacetylene. This finding prompted us to subject DCA to more exhaustive animal studies, some results of which were the subject of a preliminary communication (Reichert, Ewald & Henschler, 1971). Independent of our investigations, Siegel (1968) reported the results of animal experiments with DCA, indicating that death from pulmonary oedema had occurred 2–3 days after exposure. Further experiments (Siegel, Jones, Coon & Lyon, 1971) involved exposure to an ether adduct or a DCA/Tri mixture, rather than to DCA itself. Since our results are at variance in some respects with those of Siegel *et al.* (1971), it is considered worthwhile to describe in more detail the methodology used to prepare and analyse DCA and to determine its inhalation toxicity. This required some novel approaches not previously described.

EXPERIMENTAL

Animals. Mature female NMRI mice obtained from an animal-breeding station (P. Bäumler, D-819 Wolfratshausen) and weighing between 20 and 30 g, were used throughout the experiments. The animals were fed a standard diet (Altromin®) and had free access to tap-water. They were maintained in a room with a 12-hr light-dark cycle, atmospheric conditions being kept constant before and during the experiments (temperature 23°C and relative atmospheric humidity 70%).

Exposure of animals. The bodies of the animals were held in place in tubular, transparent PVC casings outside the actual exposure chamber, which had a dimension of 50 × 50 × 250 mm. Boreholes of appropriate size ensured that the animal tubes fitted into the gas space in such a way that only the animals' noses were in contact with the exposure mixture. This arrangement permitted exposure of ten animals at one time.

Histological examination. The organs were fixed in Bouin's solution. Sections of the liver, lung and kidneys were stained with haematoxylin–eosin, trichrome–periodic acid–Schiff, cresyl violet, Alcian Blue and colloidal iron. while haematoxylin–eosin and cresyl violet were used for the brain sections. Other staining methods used were those described by Goldner, Herxheimer, Palmgren and Gerhard.

Generation of DCA. As indicated in Fig. 1, liquid Tri was introduced into a gas-washing bottle at constant rate by means of an infusion apparatus (Perfusor, B. Braun, Melsungen) and vapourized in a thermostat (Th_1). After pre-drying over $CaCl_2$, rotametrically dosed nitrogen moved the Tri vapour across the

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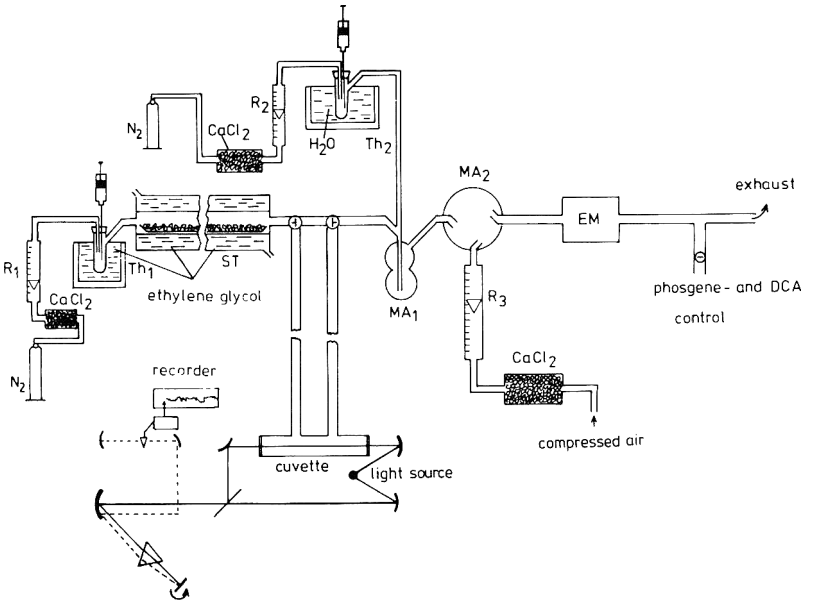

Fig. 1. Diagram of apparatus used for the preparation and analysis of defined concentrations of DCA in air for animal exposures (for explanation see text).

surface of dehydrated coarse-grained KOH, which was contained in a metal groove. A cooling jacket (Liebig condenser), perfused with ethylene glycol as the heat-exchanger, maintained the temperature in the thermostat and adjacent synthesizing tube (ST) at 120°C. The actual reaction tube had an internal diameter of 30 mm and was 1·20 m in length. Tri was converted quantitatively to DCA. By using different infusion rates for Tri, the concentration in the final exposure mixture could be varied within wide limits.

Analytical determination of DCA. From the synthesizing tube, the DCA/N_2 mixture was drawn through a 20-cm gas cuvette (NaCl press plates) of a double-beam infra-red spectrograph (Leitz, Wetzlar) and continuously recorded at a constant wavelength of 10·10 μm. DCA has a pronounced characteristic band at 10·1–10·15 μm (Fig. 2). After the DCA/N_2 mixture had passed through the infra-red cuvette, Tri vapour was added in excess for the stabilization of DCA. The Tri vapour was generated in a thermostat (Th_2) in the same way as for DCA synthesis and was fed into the DCA/N_2 stream in a mixing apparatus (MA_1). In a second mixing system (MA_2), dilution with air pre-dried over $CaCl_2$ provided the final exposure mixture (EM).

Testing for phosgene. In the presence of atmospheric oxygen, DCA decomposes rapidly to phosgene and carbon monoxide: $2ClC{\equiv}CCl + 2O_2 \rightarrow 2COCl_2 + 2CO$. Phosgene interferes decisively with DCA action in experimental animals by inducing toxic pulmonary oedema, and from the earlier reports of Siegel (1968), it appears that phosgene was present in his experiments. Bearing this in mind, we continuously checked the DCA–air mixture for the possible presence of phosgene before and after its passage through the animal exposure chamber. For this purpose we used the method outlined by Lamouroux (1956). In this method, a suction pump feeds approximately 3 litres of the exposure mixture into a washing bottle containing 20 ml of a solution of 0·1% 4-(*p*-nitroben-

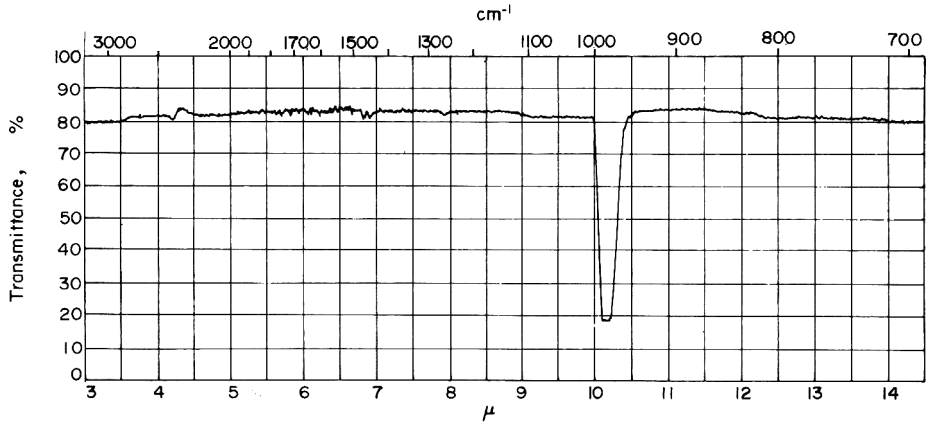

Fig. 2. Infra-red spectrum of DCA in the gaseous state, recorded with the Leitz spectrograph.

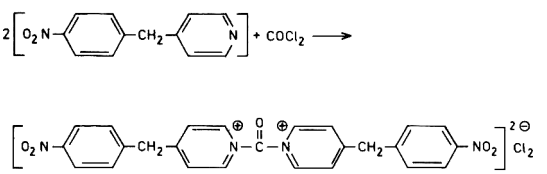

Fig. 3. Reaction of phosgene with 4-(*p*-nitrobenzyl)pyridine.

zyl)pyridine in 4-methyl-2-pentanol. The reagent rapidly forms a stable yellow-coloured complex with phosgene (Fig. 3).

The amount of Tri required to stabilize the DCA in the exposure mixture appears to have a critical limit; 60–65% of the DCA consistently decomposes to phosgene and CO once the Tri level falls below this limit (Table 1).

The infra-red absorption spectrum at 10·10 μm was used for the quantitative analysis of DCA. For standardization, particular concentrations of DCA were tested in a known volume for their chloride content by means of combustion analysis, using the apparatus described by Wickbold (1957). The relationship between the concentration of DCA (in N_2) and the infra-red absorption becomes apparent from Fig. 4. The method outlined above thus permits the measurement of the concentrations in the exposure mixture over a range of several thousand ppm to 10 ppm. Lower concentrations in the exposure mixture may be determined by direct gas-chromatographic analysis; relevant details will be reported elsewhere.

RESULTS

The LC_{50} values for 1-hr and 6-hr exposure were determined in 14 single inhalation experiments conducted in groups of ten mice. Table 2 shows the mortality rates and the average survival times. The observed signs of intoxication tended to be uncharacteristic and included an unsteady and staggering gait shortly after cessation of the exposure, deepening of respiration, pilo-erection and the absence of escape reactions; recovery time varied from a few hours to 3–4 days, depending on the exposure level.

The LC_{50} values were established as 124 (108–153) ppm for the 1-hr exposure, and as 19 (16–22·6) ppm

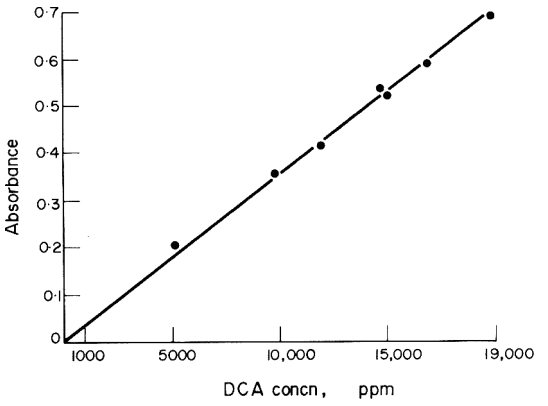

Fig. 4. Absorbance of DCA in concentrations of 1000–19,000 ppm (λ = 10·1 μm; 20-cm gas cuvette, NaCl press plates).

Table 1. *Stabilization of DCA in the air mixture using varying amounts of Tri*

DCA (ppm)	Tri ppm	Tri % equiv.	Phosgene (ppm)	DCA decomposition (%)
39·5	76·4	193	—	—
44·5	76·4	172	26·6	60
45·0	76·4	170	27·4	61
46·0	76·4	166	—	—
31·4	49·8	159	—	—
33·3	49·8	150	—	—
32·5	44·4	137	21·2	65
34·4	44·4	129	22·7	66
35·2	44·4	126	—	—
35·5	44·4	125	22·7	64
36·9	45·8	124	—	—
36·9	44·4	120	24·9	67
36·0	43·2	120	—	—
36·9	43·2	117	23·4	63

for the 6-hr exposure. The $c \times t$ rule accordingly applies for the exposure periods studied. Examination for lognormal distribution by chi-squared analysis gave no significant variation from the expected values complying with the regression line (Fig. 5).

Gross examination at autopsy provided little indication of the cause of death. The kidneys, however, appeared strikingly pale and distinctly swollen. These findings prompted another experiment, involving exposure to a sublethal dose of 101 ppm DCA for 1 hr. At 12, 24, 48 and 192 hr after termination of the exposure, groups of four animals were killed by iv injection of a lethal dose of pentobarbitone. Of the organ weights studied (lung, heart, thymus, liver and kidneys), only the kidney weights showed a marked deviation from normal (Fig. 6).

In no case was there any clear evidence of histopathological change in the lungs. Liver sections revealed fat accumulation, with slight or moderate vacuolation in the centrilobular region and distinctly basophilic cytoplasm. Pathological changes were most marked in the kidneys, where extensive necrotic lesions were observed, primarily in the mid-cortex, on day 1 of exposure. These changes involved mainly

Table 2. *Mortality and average survival time of mice exposed to varying concentrations of DCA*

Exposure time (hr)	Concn of DCA (ppm)	Mortality (%)*	Average survival time (hr)*
1	60	0	—
	77	10	138·0 ± 0·0
	91·5	40	59·4 ± 16·6
	155	50	114·0 ± 17·0
	212	90	100·2 ± 23·8
	354	100	39·1 ± 14·2
6	10	0	—
	15·6	20	83·0 ± 25·0
	18·6	60	95·8 ± 14·0
	23	80	189·4 ± 15·4
	28·6	90	90·0 ± 29·5
	42·8	100	99·4 ± 22·5

*Ten animals per exposure group.

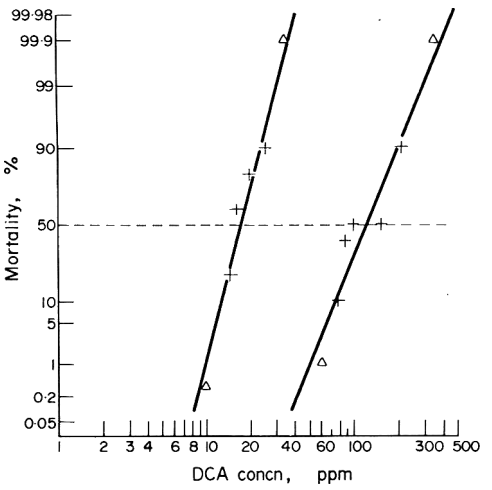

Fig. 5. Regression lines obtained on determination of the LC_{50} in the 1-hr (a) and 6-hr (b) exposure.

the distal segments of the proximal tubule. Besides numerous necrotic foci, regions with clearly flattened epithelium and distinctly basophilic cytoplasm were found, in association with an increase in mitotic activity, after 2 days. The number and intensity of the necrotic foci decreased markedly after 8 days, but numerous mitotic figures continued to appear. Fat- and PAS-positive epithelial cells had sloughed into the tubular lumen.

The brain sections revealed generalized tissue oedema and axonal swelling, together with hyperchromatically atrophied cells in the brain stem. The ganglion cells in the cerebral cortex and in the Purkinje layer of the cerebellum had shrunk. A more detailed description and analysis of the patho-anatomical lesions observed in the kidney and brain in the course of more exhaustive experiments will be the subject of separate communications.

DISCUSSION

The procedure for preparing DCA from Tri, the principle of which has been described by Ott, Ottemeyer & Packendorff (1930), was found to be a useful basis for studying the toxicological profile of the material. The difficulties involved in preparing low DCA concentrations, which had to be kept constant over prolonged periods were overcome by appropriate modification of the apparatus, but the use of DCA in animal experiments depends on the addition of a stabilizer. DCA is highly reactive and immediately enters into a violent exothermal reaction with atmospheric oxygen to form phosgene and carbon monoxide. Ether (Ott, 1931; Siegel *et al.* 1971) and Tri (Reichert, Brinke, Bannasch & Liebaldt, 1972; Reichert *et al.* 1971; Siegel *et al.* 1971) proved to be of advantage as stabilizing agents.

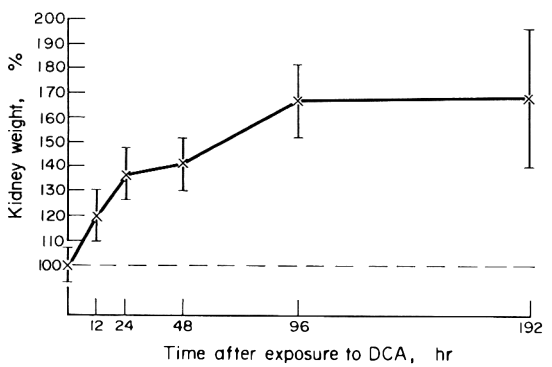

Fig. 6. Mean kidney weights for groups of four animals killed at intervals of 12–192 hr after exposure to 101 ppm DCA for 1 hr.

DCA–ether mixtures are of interest for the preparation, storage and use of relatively large amounts of the explosive compound; they have so far not attained any major toxicological significance, in that poisonings with this molecular compound (composition 1:1) have not been reported in the literature. The biological activity of DCA in this compound is clearly depressed (Siegel *et al.* 1971) and it is possible that there are qualitative differences in action as well. We stabilized DCA, therefore, with Tri. The amount of Tri incorporated in the mixture is decisively influenced by equipment constants, such as atmospheric humidity, the moisture content of KOH and the rapidity with which blending of the DCA/Tri/N_2/air mixture is achieved in the mixing bell (MA_2 in Fig. 1). Our experiments required a two- to fourfold excess of Tri over DCA. When inhaled simultaneously at such low concentrations, Tri itself is not likely to exert any toxic effects.

Saunders (1967). who did not observe decomposition of DCA in the air when using low concentrations, put the upper limit of stability at 10 ppm. Our findings failed to support his results, however, as we consistently found decomposition of DCA in this range.

During the 4 yr in which our apparatus has been in operation under the conditions described, no spontaneous ignition or explosion of DCA has occurred either inside the apparatus or outside. Our technique can be considered safe, therefore, for the administration of DCA in animal experiments.

As regards the toxic potential of DCA in mice, there are two results which are noteworthy. The lung tissue remained completely unaffected by DCA and there was, in particular, no evidence of pulmonary oedema. The pulmonary oedema found by Siegel (1968) was most probably due to contamination with phosgene. The second striking observation in our experiments was that DCA caused severe kidney lesions which were clearly the cause of death in the mice. This nephrotoxic action has not been demonstrated in man, the selective lesions of the cranial nerves being the most prominent feature in cases of human intoxication. Buxton & Hayward (1967) noted some shrinkage of the cells of the distal tubules, but attributed this to post-mortem changes. Clinically they had diagnosed only some transitory renal impairment in the form of moderate albuminuria, glycosuria (0·25%), numerous oxalate crystals in the urine and an increase in blood urea to 65 mg/100 ml. In their description of a severe case of DCA poisoning, Henschler *et al.* (1970) reported only a slight inhibition of renal concentrating capacity, the other renal findings being in the normal range. Similarly, other case reports include no important data suggestive of

impaired renal function. The intoxication picture in man is consistently characterized by severe neurological symptoms.

After studying the effects of DCA in different concentrations and after varying exposure periods Jackson, Lyon & Siegel (1971) and Siegel *et al.* (1971) described only an increase in cellular and nuclear size with partial clumping of the nuclear chromatin in the epithelial cells of the proximal tubules, but in no case was there any evidence of widespread tubular necrosis such as we observed.

The considerable discrepancy between the pathomorphological findings in animal experiments and the largely normal renal findings in human intoxications calls for further clarification. Such studies should include blood chemistry and morphological controls to elucidate the cause and extent of the nephrotoxic effect of DCA.

As far as the neurotoxic action of DCA is concerned, reports in the literature detailing experimental findings are sparse and incomplete. Humphrey & McClelland (1944) gave a cursory report of numerous focal necroses with generalized glial proliferation in the brain, in association with an encephalitic reaction of the brain membranes, in two rabbits exposed, by means of an anaesthetizing device, to mixtures of Tri or ether and an unknown concentration of DCA. In contrast, the mice used in our experiments exhibited non-specific changes in various brain regions, furnishing some general indication of a neurotoxic effect of DCA without pointing to the presence of distinct cranial nerve lesions as described in human patients. Our findings in mice should, however, be assessed with some caution in view of the co-existence of uraemia and the possibility that the mode of killing (a high iv dose of pentobarbitone) may have led to certain non-specific neurological alterations. For future studies of this problem, it is suggested that larger animals should be used and that the neuropathological pattern in the central and peripheral nervous systems should be examined systematically in programmed time-course studies.

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THE EFFECT OF CTAB, A CATIONIC SURFACTANT, ON THE ABSORPTION RATE OF [^{14}C]TRIPALMITATE FROM A TEST MEAL IN THE RAT

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Abstract—The effect of CTAB, a cationic surfactant, on the absorption rate of [^{14}C]tripalmitate from a test meal was studied in male rats. It was found that the surfactant in small doses (0·8, 2·4 or 8·0 mg/kg) increased the absorption rate of [^{14}C]tripalmitate from the intestinal tract by accelerating gastric emptying. However, at a dose of 400 mg/kg a marked retardation of gastric emptying was observed. When administered intragastrically to pylorus-ligated rats, the surfactant did not alter the amount of fluid secreted into the stomach.

INTRODUCTION

Surfactants have been found by numerous workers to influence the rate and extent of gastro-intestinal absorption of drugs and nutrient substances, enhancement as well as inhibition of such absorption having been observed. In several reports, alterations in gastro-intestinal absorption in the presence of surfactants have been ascribed to interaction between drug and surfactant (micellar solubilization and complex formation). Some surfactants, however, have been found to modify the membrane permeability in concentrations below the critical micellar concentration (Feldman & Gibaldi, 1969; Levy, Miller & Reuning, 1966; Moore, Zatzman & Overack, 1971; Penzotti & Mattocks, 1968). Moreover, surfactants may influence the rates of gastric emptying and intestinal transit which, in turn, may influence intestinal absorption (Feldman, Wynn & Gibaldi, 1968; Lish, 1961; Necheles & Sporn, 1966).

Most reports on the influence of surfactants on gastro-intestinal absorption have been concerned with nonionic and anionic surfactants, only a few studies on this aspect of cationic surfactants having been reported. Cationic surfactants are widely used as germicides and santizing agents under conditions in which they may possibly be ingested by man. In perfusion experiments with rat and rabbit intestines, Nissim (1960) found a marked depression of intestinal absorption of glucose, methionine and sodium butyrate from solutions containing 0·01 or 0·1% cetyltrimethylammonium bromide (CTAB). At the higher concentration, injury to the intestinal villi was observed, but the author suggested that specific enzyme actions might be involved in the inhibition of absorption. Taylor (1963) studies the effect of CTAB on transport and metabolism in sacs of everted rat intestine. At a concentration of 10^{-4} M (36 ppm), CTAB caused no histological damage to the everted intestine, but at 5×10^{-4} M and 10^{-3} M there was considerable injury to the mucosa. Transport of glucose, methionine and water was inhibited at concentrations of $2{\cdot}5 \times 10^{-4}$ M and above. An increase in passive intestinal permeability to glucose *in vitro* in the presence of CTAB (50–500 ppm) was observed by Moore *et al.* (1971). No histological injury to the mucosal epithelium was found with concentrations below 250 ppm. Kakemi, Sezaki, Muranishi & Matsui (1967) found, in rabbits, an increase in rectal absorption of sulfisoxazole from an oily base containing 0·1–1·0% CTAB. This stimulating effect on drug absorption was not observed with nonionic or anionic surfactants. With higher concentrations of CTAB, however, a reduction in sulfisoxazole absorption was observed.

This paper deals with the influence of CTAB on the *in situ* absorption rate of tripalmitate from a test meal given to rats.

EXPERIMENTAL

Chemicals. CTAB, 99% pure, was obtained from E. Merck AG, Darmstadt, Germany. Glyceryl tri[1-^{14}C]palmitate was purchased from the Radiochemical Centre, Amersham, Bucks. The specific activity of the compound was 57 μCi/mg and the radiochemical purity was 99%.

Experimental procedures. Male Sprague–Dawley rats (200–250 g) were used in all experiments. The animals were starved for 24 hr prior to gastric intubation. Normal laboratory pellet food (fat content 3·5%) was thoroughly homogenized in benzene, and glyceryl tri[1-^{14}C]palmitate dissolved in benzene was added. The benzene was evaporated in a water-bath at about 50°C and an appropriate aqueous solution of CTAB was added (2·3 ml/g laboratory food). The test meal (12·0 g/kg) was introduced into the stomach by intubation. The administered radioactivity was about 2 μCi/rat and the levels of CTAB in the intubated meal were equivalent to a dose of 0·8, 2·4, 8·0 or 400 mg/kg body weight.

The rats were killed 4 or 8 hr after gastric intubation and an abdominal incision was made to expose

the gastro-intestinal tract. The gastro-oesophageal junction, pylorus, ileo-caecal valve and rectum were clamped and the gastro-intestinal tract was removed and stripped of adhering fat. The small intestine was divided into two parts of equal length and the contents of the different parts of the gastro-intestinal tract were collected by rinsing with saline. The various samples of gastro-intestinal contents, the stomach, the two parts of the small intestine, the caecum and the colon were each placed in a solution containing equal quantities of 40% sodium hydroxide and ethanol, and were incubated at 55°C for 12 hr. Aliquots (0·2–0·3 g) of the dissolved samples were pipetted into counting vials and 10 ml of a scintillation fluid (680 ml toluene, 320 ml ethanol, 5·88 g 2·5-diphenyloxazole (PPO) and 0·12 g *p*-bis-(*o*-methylstyryl)benzene) was added. All samples were counted in a liquid scintillation spectrometer (LKB-Wallac 81000, LKB-Wallac, Turku) and the values were corrected for quenching by internal standardization.

For the study of gastric secretion, rats were starved for 24 hr and water was withdrawn 2 hr prior to the experiment. The animals were anaesthetized with sodium pentobarbitone (40 mg/kg, ip) and an abdominal incision was made to expose the stomach. The stomach was ligated at the pylorus and solutions of CTAB (in normal saline) were injected into the stomach through the region of the forestomach. Control animals received saline. The injected volume in all cases was 4·0 ml/kg body weight and the doses of CTAB injected were equivalent to 0·4, 80 or 400 mg/kg body weight. The abdominal incision was closed and the animals were killed 1 hr after the injection. The stomach was removed and the weight of the contents was recorded.

RESULTS

Table 1 shows the recovery of radioactivity from the different parts of the gastro-intestinal tract 4 and 8 hr after the administration of the test meal. The extent of absorption was calculated by assuming that the material not recovered from the gut and its contents had been absorbed, while gastric emptying was expressed directly as the percentage of administered radioactivity no longer retained by the stomach and its contents. Rats given CTAB in a dose of 8·0 mg/kg (0·067% CTAB in the test meal) showed a significant increase in gastric emptying 8 hr after the gastric intubation. In rats given 400 mg/kg (3·33% in the test meal), however, a marked decrease in gastric emptying was observed. At this dose the tone of the gastric muscles appeared to be markedly reduced. Only 32% of the administered radioactivity (compared with 85% in the control) had left the stomach within 8 hr. In rats killed 4 hr after gastric intubation of 0·8, 2·4 or 8·0 mg CTAB/kg there was a dose-related increase in the rate of gastric emptying (Table 1) and at 2·4 and 8·0 mg/kg, gastric emptying was significantly higher than in control animals.

With an increase in the rate of gastric emptying, there was a corresponding increase in the amount of [^{14}C]tripalmitate absorbed. The amount of tripalmitate absorbed, expressed as a fraction of the amount that had left the stomach, was approximately the same in all groups, suggesting that the CTAB did not interfere with the mechanism of fat absorption.

The only significant differences between control and treated animals in the amounts of radioactivity recovered from the gastro-intestinal wall were found in animals receiving 400 mg CTAB/kg. In these animals the amount of radioactivity found in the wall

Table 1. *Amount of radioactivity in different parts of the gastro-intestinal tract 4 or 8 hr after oral administration of a test meal containing [^{14}C]tripalmitate and CTAB*

Region of gut	Wall (W) or contents (C)	Amount of radioactivity (% of administered dose) following administration of CTAB in a dose (mg/kg) of 0	0·8	2·4	8·0	400
		At 4 hr				
Stomach	W	3·93 ± 1·33	2·67 ± 0·78	2·29 ± 1·09	1·88 ± 0·20	—
	C	42·29 ± 5·29	34·07 ± 3·19	25·59 ± 2·85*	20·72 ± 6·52*	—
Small intestine:						
First half	W	4·83 ± 2·28	4·99 ± 1·43	2·89 ± 0·36	3·44 ± 1·03	—
	C	4·01 ± 1·17	4·01 ± 2·55	6·66 ± 1·92	4·58 ± 0·96	—
Second half	W	0·47 ± 0·07	0·97 ± 0·27	0·62 ± 0·07	0·52 ± 0·16	—
	C	1·06 ± 0·25	1·65 ± 0·12	1·95 ± 0·54	1·12 ± 0·25	—
Caecum	W	0·10 ± 0·01	0·19 ± 0·02	0·14 ± 0·04	0·17 ± 0·04	—
	C	0·37 ± 0·23	0·27 ± 0·15	0·58 ± 0·17	0·93 ± 0·28	—
Colon	W	0·10 ± 0·01	0·15 ± 0·01	0·14 ± 0·02	0·13 ± 0·03	—
	C	0·06 ± 0·01	0·11 ± 0·02	0·07 ± 0·03	0·07 ± 0·01	—
	Gastric emptying†	53·78 ± 5·98	63·26 ± 3·88	72·12 ± 3·69*	77·40 ± 6·52*	—
	Absorption‡	42·78 ± 6·22	50·92 ± 3·18	59·07 ± 3·61	66·44 ± 4·45*	—
		At 8 hr				
Stomach	W	1·13 ± 0·07	—	—	0·64 ± 0·12	1·73 ± 0·41
	C	13·89 ± 2·76	—	—	5·11 ± 0·65*	66·13 ± 2·85***
Small intestine:						
First half	W	0·97 ± 0·11	—	—	1·16 ± 0·12	0·58 ± 0·06*
	C	5·18 ± 0·76	—	—	4·84 ± 1·00	3·62 ± 1·38
Second half	W	0·48 ± 0·10	—	—	0·35 ± 0·08	0·22 ± 0·01*
	C	1·30 ± 0·35	—	—	0·95 ± 0·27	0·34 ± 0·10*
Caecum	W	0·10 ± 0·02	—	—	0·08 ± 0·02	0·08 ± 0·01
	C	1·22 ± 0·19	—	—	0·85 ± 0·27	0·36 ± 0·09**
Colon	W	0·12 ± 0·03	—	—	0·11 ± 0·03	0·08 ± 0·01
	C	0·06 ± 0·02	—	—	0·17 ± 0·08	0·10 ± 0·02
	Gastric emptying†	84·98 ± 2·83	—	—	94·25 ± 0·56*	32·14 ± 3·21***
	Absorption‡	75·55 ± 2·34	—	—	85·74 ± 1·56**	26·76 ± 4·39***

†Percentage of administered radioactivity not retained in stomach wall and contents.
‡Percentage of administered radioactivity not recovered from entire gastro-intestinal tract and contents.
Values are means ± SEM for groups of three or four rats and those marked with asterisks differ significantly (Student's *t* test) from the corresponding control value: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

of the small intestine was lower than that in control animals, a probable reflection of the delay in gastric emptying.

CTAB did not alter the amount of fluid secreted into the stomach in pylorus-ligated rats when administered intragastrically in a dose of 0·4, 4 or 80 mg/kg (Table 2). At 400 mg/kg there was a slight but not statistically significant increase in the pH of the gastric contents and in the amount of fluid secreted into the stomach.

Table 2. *Weight and pH of gastric contents of rats 1 hr after an intragastric injection of 0·4–400 mg CTAB/kg body weight in 4 ml aqueous solution/kg*

Dose of CTAB (mg/kg)	Gastric contents	
	Weight (g/100 g body weight)	pH
0	2·60 ± 0·55	1·73 ± 0·14
0·4	2·37 ± 0·26	1·65 ± 0·02
4·0	2·75 ± 0·34	1·66 ± 0·08
80	2·36 ± 0·05	1·76 ± 0·03
400	3·45 ± 0·29	2·47 ± 0·38

Values are means ± SEM for groups of three or four rats.

DISCUSSION

As previously noted, surfactants may influence the absorption rate of drugs and nutrient substances in several ways. In this work, we studied the influence of CTAB, a cationic surfactant, on the absorption rate of [^{14}C]tripalmitate from a test meal. It was found that CTAB in small doses (0·8, 2·4 or 8·0 mg/kg) increased the absorption rate of tripalmitate by increasing the rate of gastric emptying. The latter rate increase appeared to be proportional to the amount of CTAB administered. This enhancement of gastric emptying occurred at doses far below the lethal doses reported for alkyltrimethylammonium surfactants, oral LD_{50}s in the range of 250–1000 mg/kg having been reported for these compounds (Cutler & Drobeck, 1970). However, at a dose of 400 mg/kg a marked decrease in gastric emptying was observed and at this dose the tone of the gastric muscles appeared to be markedly reduced.

Previous studies (Feldman *et al.* 1968; Hunt & Macdonald, 1954) have shown that drugs can influence the rate of gastric emptying by altering gastric secretion. Feldman *et al.* (1968) observed a depressed rate of gastric emptying of phenol red when sodium deoxycholate was given orally to rats and suggested that this decrease in gastric emptying was due to a stimulation of the gastric secretion by the bile salt. It has also been shown that as the volume of a liquid test meal increases, the percentage of gastric contents leaving the stomach per unit time decreases (Hunt & Macdonald, 1954). However, at doses that increased the rate of gastric emptying, CTAB did not alter the amount of fluid secreted into the stomach, and the slight increase in gastric secretion associated with the highest dose (400 mg CTAB/kg) was not enough to explain the marked inhibition of gastric emptying at this dose. In this connexion, however, it should be noted that CTAB was administered intragastrically as an aqueous solution in this study of gastric secretion but was given together with nutrient substances in the absorption study, and the dose levels are therefore not directly comparable. Cationic surfactants like CTAB have a great affinity for negatively charged molecules and substances and part of the orally administered CTAB was probably bound to substances in the test meal.

Surfactants have been found to affect the motility of the gastro-intestinal tract. Nicheles & Sporn (1966) observed an inhibition of gastric motility in dogs following the introduction of surfactants (100–500 mg) into the gastric pouch. Dioctyl sulphosuccinate, an anionic surfactant, was found by Lish (1961) to decrease the rate of propulsion of a test meal through the gastro-intestinal tract when the surfactant was administered orally in a dose of 400 or 1600 mg/kg to rats. Sasaki (1954a,b), on the other hand, found in rabbits an increase in gastric and intestinal motility when bile salts were given orally in small doses (20–40 mg/kg) but a decrease in motility with higher dose levels. It is therefore tempting to assume that CTAB in small doses increases the rate of gastric emptying by increasing gastric motility. Sasaki (1954a) suggested that the bile salts acted directly on the muscles of the gastro-intestinal tract and not through the autonomic nervous system. This may hold also for the cationic surfactant CTAB. Alternatively, the surfactant may act by liberating hormones from the gastro-intestinal mucosa. The present data do not permit any distinction between these two possibilities.

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LONG-TERM TOXICITY OF FOUR FLUORESCENT WHITENING AGENTS

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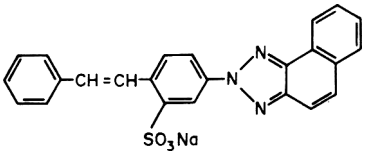
Abstract—Sodium 2-(4-styryl-3-sulphophenyl)-2*H*-naphtho[1,2-*d*]triazole (I), disodium 4,4'-bis-[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)amino]stilbene-2,2'-disulphonate (II), disodium 4,4'-bis-([4-anilino-6-(*N*-methyl-2-hydroxyethylamino)-1,3,5-triazin-2-yl]amino)stilbene-2,2'-disulphonate (III) or disodium 4,4'-bis-(2-sulphostyryl)biphenyl (IV) fed daily to albino rats for 2 yr at dietary levels of 40, 200 or 1000 ppm produced no adverse effects. Fibrous peritonitis was noted in some beagle dogs fed various levels of compound I over a 2-yr period. This finding represented the healing stage of an inflammatory process, which apparently resulted from the presence of residual caustic soda from a manufacturing step when unbuffered compound I was fed to the dogs for the first 30 wk of the 2-yr study. There were no other significant observations definitely attributable to the ingestion of the material. Compounds II, III and IV had no adverse effects when fed to dogs for 2 yr at dietary levels up to 2000 ppm. Accumulation of the compounds in the dog tissues was negligible. When fed to male and female albino rats throughout a three-generation reproduction study at dietary levels up to 1000 ppm, none of the compounds caused any toxic effects. The reproductive performance of the animals was not adversely affected, and no malformations were observed in the progeny. No histopathological changes were seen in parental animals from each generation or in the F_{3b} progeny.

INTRODUCTION

Fluorescent whitening agents (FWAs) or optical brighteners are organic compounds used in detergents to whiten the products and to impart desirable whiteness to washed fabrics. Keplinger, Fancher, Lyman & Calandra (1974) recently reported on the toxicity testing of the four FWAs shown in Fig. 1. All were found to be of low acute and subacute toxicity and were neither teratogenic nor mutagenic. A 2-yr feeding study in rats and dogs and a multigeneration reproduction study in rats were described in the paper as being in progress. The completed results of the latter studies are reported here, as well as of analyses for the four FWAs in the tissues of dogs fed the compounds for 90 days and 2 yr as described by Keplinger *et al.* (1974). All test materials were taken from commercial production.

Compound No. I



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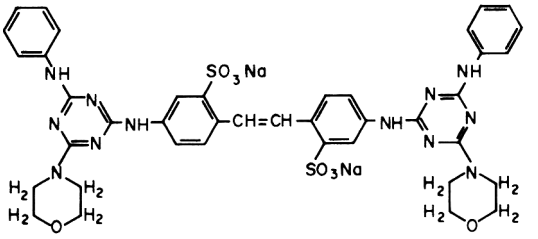

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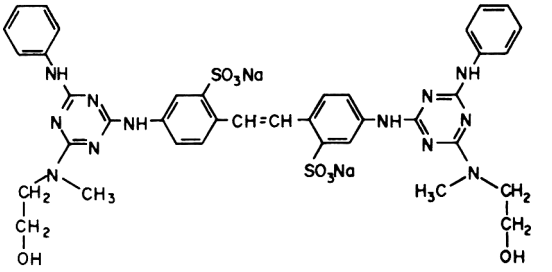

Compound No. IV

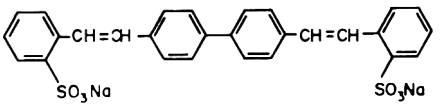

Fig. 1. Chemical structures of four fluorescent whitening agents: I—Tinopal® RBS, sodium 2-(4-styryl-3-sulphophenyl)-2*H*-naphtho[1,2-*d*]triazole; II—Tinopal AMS, disodium 4,4'-bis[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)amino]stilbene-2,2'-disulphonate; III—Tinopal 5BM, disodium 4,4'-bis-([4-anilino-6-(*N*-methyl-2-hydroxyethylamino)-1,3,5-triazin-2-yl]amino)stilbene-2,2'-disulphonate; IV—Tinopal CBS, disodium 4,4'-bis-(2-sulphostyryl)biphenyl. Compounds I, II and III have been designated by the American Society for Testing and Materials as NTS-1, DASC-3 and DASC-4, respectively. Tinopal® is a registered trade-name of CIBA-GEIGY, Inc.

EXPERIMENTAL

Tissue residues. Tissue samples were taken from representative male and female dogs used in the 90-day oral toxicity study reported by Keplinger *et al.* (1974) and from the 2-yr oral toxicity study reported in this paper. Tissue homogenates of liver, muscle, fat, kidney and brain were prepared with water containing an internal fluorescent standard. With the exception of fat, each homogenate was extracted twice by ultrasonication in an acetone–water–concentrated ammonium hydroxide mixture (90 : 10 : 5, by vol.). Solid NaCl was added to aid in the extraction. The extracts were clarified by centrifugation, concentrated and subjected to thin-layer chromatography (TLC) on silica gel 60 plates (No. 5763, EM Laboratories), along with appropriate standards. Homogenized fat samples were extracted with a mixture of chloroform, methanol and concentrated ammonium hydroxide (60 : 40 : 10, by vol.) and after centrifugation, the upper layers were removed and chromatographed. For compound I, the TLC plates were developed in a solvent system consisting of dioxane–benzene–methanol–concentrated ammonium hydroxide (40 : 50 : 20 : 10, by vol.), while for II, III and IV, the solvent system used was acetone–water–concentrated ammonium hydroxide–tetrabutylammonium iodide (90:4:10:20, by vol.). Following TLC, test agents present on the plates were quantitated by thin-layer reflectance densitometry using a Hitachi-Perkin Elmer MPF-2A spectrofluorometer filled with a thin-layer plate-scanning accessory. At least three replicate control samples of each tissue and of blood were separately spiked with 100–500 ng/g (or ml) of each FWA. These samples were then taken through the method and recoveries were determined. Recovery was generally greater than 90%. The sensitivity of the method was 0·02 μg/g (ppm).

Long-term (2-yr) oral toxicity. Each of the four materials was fed to albino rats and beagle dogs as described by Keplinger *et al.* (1974). Rats received 40, 200 or 1000 ppm daily, equivalent to 4, 20 or 100 mg/kg body weight/day. Dogs ingested 80, 400 or 2000 ppm, equivalent to 2, 10 or 50 mg/kg/day. Compound I was fed unbuffered to dogs at dietary levels of 20, 70 or 200 ppm for the first 30 wk, and the dosage was then raised, over a period, to levels of 80, 400 or 1000 ppm, using test material neutralized to pH 7 before admixture with the stock diet. At termination of the study, the animals were killed and tissues from the dogs given the highest dietary level of the FWAs were analysed for FWA content in the manner previously described. Blood samples were also collected from the dogs and analysed for FWA content. For this, each blood sample was mixed with water containing an internal fluorescent standard and

Table 1. *Blood and tissue levels of four FWAs at the termination of 90-day and 2-yr oral toxicity studies in beagle dogs*

Test material	Dietary level (ppm)	Sex	FWA concentration (ppm) Blood*	Brain*	Kidney*	Liver*	Muscle*	Fat*
			After 90 days					
Compound I	1000	M	—	ND	ND	0·2	ND	TR
		F	—	ND	ND	TR	ND	ND
Compound II	10.000	M	—	ND†	ND	ND	0·1	0·5
		F	—	TR†	ND	ND	0·2	1·2
Compound III	10.000	M	—	ND	ND	ND	0·9	0·6
		F	—	ND	ND	ND	TR	0·4
Compound IV	10.000	M	—	ND	TR	0·3	0·2	0·7
		F	—	ND	TR	0·2	ND	0·3
			After 2 yr					
Compound I	1000	M	0·02	ND	0·08	0·35	ND	0·02
		M	0·04	ND	0·03	0·34	ND	0·03
		F	0·02	ND	0·04	0·34	ND	0·02
		F	0·15	ND	0·02	0·19	0·04	0·05
Compound II	2000	M	0·04	0·04	ND	ND	0·09	0·08
		M	0·01	ND	TR	TR	TR	0·05
		F	0·02	ND	TR	TR	TR	TR
		F	0·01	ND	TR	TR	TR	0·03
Compound III	2000	M	TR	ND	ND	TR	ND	ND
		M	TR	ND	0·05	ND	ND	ND
		F	TR	ND	0·06	ND	ND	ND
		F	TR	ND	0·09	TR	ND	TR
Compound IV	2000	M	0·01	ND	0·02	0·26	ND	0·10
		M	0·01	ND	0·14	0·63	TR	0·06
		F	0·01	ND	0·08	0·28	ND	0·07
		F	0·01	ND	0·06	0·51	TR	0·06

ND = None detected, by methods sensitive to 0·01 ppm in blood and 0·02 ppm in tissues
TR = Trace detected, insufficient for quantitative estimation
*No FWA residues were detected in tissues from control animals fed FWA-free diet for 90 days (one male and one female) or 2 yr (two males and two females).
†Tissue derived from dogs given 2000 ppm Compound II daily for 90 days.

was extracted with ethyl acetate–methanol (1 : 1, v/v). The extracts were removed, concentrated and finally reconstituted for TLC analysis as described above. Sensitivity of this procedure was 0·01 μg/ml (ppm).

Three-generation reproduction study. The procedure used was that of Kennedy, Fancher & Calandra (1968), the test materials being given to albino rats at dietary levels of 40, 200 and 1000 ppm throughout the study.

RESULTS

Tissue residues in beagle dogs

Tissue concentrations noted after ingestion of the FWAs for 90 consecutive days and for 2 yr are shown in Table 1. When compared with the total quantity ingested, tissue accumulation of the test materials was negligible.

Long-term (2-yr) oral toxicity

Albino rats. Daily feeding of compounds I–IV at dietary levels up to 1000 ppm caused no abnormalities in body weights, survival, behaviour, haematology, clinical chemistry or urine analysis. No abnormalities were observed during gross and microscopic pathological examination.

Beagle dogs. No effects on any parameter were observed among male and female dogs given 80 ppm compound I in the diet. Diminished body-weight gains were noted in two (females) of eight dogs given 400 ppm, and in one male and two females at the 1000 ppm level. One female at each of these dietary levels had a depressed erythrocyte count, haemoglobin concentration and haematocrit, 6–9 months after feeding was initiated. A rise in serum alkaline-phosphatase activity was found at months 9 and 12 in the same female given 400 ppm and also in a different female in the 1000 ppm group at months 9, 12 and 18. However, no significance was attached to these findings since the haematological values and serum alkaline-phosphatase levels were normal at the conclusion of the study.

The results of other clinical chemistry parameters were not noteworthy. Blood urea nitrogen, serum glucose, serum glutamic–oxalacetic and glutamic–pyruvic transaminase levels and urine analysis values were within the normal range throughout the study.

No deaths occurred in dogs given any dietary level of compound I, but autopsy of animals killed at the termination of the study revealed large amounts of abdominal serous fluid in some animals in the 400 and 1000 ppm groups. The abdominal viscera of these animals had fibrous adhesions involving the serosal surface. Microscopically, the gastro-intestinal tissue showed organized granulation of inflammatory origin. The findings are suggestive of chronic or fibrous peritonitis. However, there was no evidence of perforations in the wall of the abdominal viscera or of mucosal lesions which might explain the aetiology or pathogenesis of the peritonitis. Since the compound was fed unbuffered during the first 30 wk, it is possible that the peritonitis could have resulted from an alkalinity effect during this part of the study. Splenic

Table 2. *Effects of dietary levels of 0–1000 ppm compound I on the reproductive indices of three generations of rats*

Dietary level (ppm)	Generation	Mating index*	Fertility index*	Pregnancy index*	Mean litter size	Live-birth index*	5-Day survival index*	Lactation index*
	F_{1a}	72·7	100·0	100·0	12·1	91·8	94·9	86·2
	F_{1b}	60·7	88·2	93·7	12·3	99·5	89·6	96·2
	F_{2a}	35·6	100·0	100·0	8·4	88·9	62·5	73·6
	F_{2b}	55·2	100·0	100·0	10·2	95·7	76·3	55·7
	F_{3a}	30·2	75·0	80·0	11·1	94·7	59·5	57·4
	F_{3b}	35·7	100·0	90·0	12·3	100·0	78·4	88·3
40	F_{1a}	54·5	83·3	93·7	11·5	99·4	97·7	91·7
	F_{1b}	75·0	93·3	93·3	13·6	99·5	85·8	82·1
	F_{2a}	44·1	93·3	93·3	9·1	98·4	67·5	67·5
	F_{2b}	63·6	92·9	92·9	11·4	100·0	90·5	64·9
	F_{3a}	32·6	92·9	81·3	10·0	92·2	61·2	60·0
	F_{3b}	60·0	80·0	100·0	8·8	98·1	84·6	95·1
200	F_{1a}	60·7	94·1	100·0	11·1	97·7	99·4	86·9
	F_{1b}	84·2	100·0	100·0	12·9	100·0	90·8	81·9
	F_{2a}	45·7	100·0	100·0	10·1	98·7	80·0	52·1
	F_{2b}	59·2	100·0	100·0	10·1	100·0	81·4	58·5
	F_{3a}	37·2	87·5	87·5	11·5	90·7	71·3	62·8
	F_{3b}	39·4	100·0	92·9	11·3	99·3	71·6	73·2
1000	F_{1a}	53·3	100·0	100·0	11·2	98·9	98·9	86·0
	F_{1b}	85·7	88·9	100·0	12·1	98·5	78·0	78·6
	F_{2a}	53·3	100·0	100·0	10·4	98·2	46·6	53·5
	F_{2b}	53·3	93·7	93·7	11·1	98·8	55·8	70·0
	F_{3a}	36·2	94·1	93·8	9·3	94·2	68·7	69·4
	F_{3b}	55·6	100·0	100·0	11·0	100·0	74·5	93·5

*Mating index = (copulations/oestrous cycles) × 100; fertility index = (pregnancies/copulations) × 100; pregnancy index = (pregnancies/females mated) × 100; live-birth index = (pups born viable/total pups delivered) × 100; 5-day survival index = (pups viable at day 5/pups born viable) × 100; lactation index = (pups viable at day 21/pups retained at day 5) × 100.

Table 3. *Effects of dietary levels of 0–1000 ppm compound II on the reproductive indices of three generations of rats*

Dietary level (ppm)	Generation	Mating index*	Fertility index*	Pregnancy index*	Mean litter size	Live-birth index*	5-Day survival index*	Lactation index*
0	F_{1a}	72·7	100·0	100·0	12·1	91·8	94·9	86·2
	F_{1b}	60·7	88·2	93·7	12·3	99·5	89·6	96·2
	F_{2a}	35·6	100·0	100·0	8·4	88·9	62·5	73·6
	F_{2b}	55·2	100·0	100·0	10·2	95·7	76·3	55·7
	F_{3a}	30·2	75·0	80·0	11·1	94·7	59·5	57·4
	F_{3b}	35·7	100·0	90·0	12·3	100·0	78·4	88·3
40	F_{1a}	84·2	100·0	100·0	12·6	99·0	93·5	94·6
	F_{1b}	73·9	94·1	100·0	13·1	100·0	75·6	84·7
	F_{2a}	53·3	100·0	100·0	9·5	96·7	53·7	58·9
	F_{2b}	57·1	93·7	93·7	10·3	96·1	53·7	72·7
	F_{3a}	41·0	93·8	93·8	9·8	93·9	91·3	76·7
	F_{3b}	69·6	93·8	100·0	11·7	98·9	80·9	95·9
200	F_{1a}	50·0	100·0	93·7	12·0	96·7	86·8	87·8
	F_{1b}	55·6	93·3	93·3	14·1	99·5	73·6	85·2
	F_{2a}	41·0	100·0	100·0	11·5	95·7	74·7	50·0
	F_{2b}	88·9	100·0	100·0	11·2	99·4	61·2	71·9
	F_{3a}	51·4	77·8	87·5	9·3	95·4	78·2	54·9
	F_{3b}	48·1	84·6	84·6	9·0	92·6	77·3	94·3
1000	F_{1a}	63·0	94·1	100·0	13·4	98·1	95·7	86·1
	F_{1b}	85·0	94·1	100·0	13·2	98·6	79·3	83·1
	F_{2a}	76·2	100·0	100·0	11·7	97·9	77·7	57·3
	F_{2b}	66·7	100·0	100·0	11·7	95·7	83·2	80·5
	F_{3a}	43·2	87·5	87·5	10·8	94·3	68·9	76·3
	F_{3b}	44·8	100·0	92·8	12·2	99·4	96·2	88·2

*Mating index = (copulations/oestrous cycles) × 100; fertility index = (pregnancies/copulations) × 100; pregnancy index = (pregnancies/females mated) × 100; live-birth index = (pups born viable/total pups delivered) × 100; 5-day survival index = (pups viable at day 5/pups born viable) × 100; lactation index = (pups viable at day 21/pups retained at day 5) × 100.

Table 4. *Effects of dietary levels of 0–1000 ppm compound III on the reproductive indices of three generations of rats*

Dietary level (ppm)	Generation	Mating index*	Fertility index*	Pregnancy index*	Mean litter size	Live-birth index*	5-Day survival index*	Lactation index*
0	F_{1a}	56·6	94·1	100·0	11·3	100·0	98·3	92·7
	F_{1b}	100·0	100·0	100·0	11·5	98·9	98·4	98·0
	F_{2a}	48·6	88·2	93·7	10·7	95·6	90·8	85·2
	F_{2b}	53·6	93·3	100·0	11·5	97·5	86·6	76·9
	F_{3a}	57·1	94·1	100·0	10·4	100·0	97·6	68·9
	F_{3b}	80·9	94·1	100·0	10·8	95·9	95·8	84·7
40	F_{1a}	47·8	68·1	93·7	12·3	93·0	95·0	75·6
	F_{1b}	77·8	87·5	100·0	12·4	99·4	87·9	86·4
	F_{2a}	44·4	100·0	100·0	10·9	98·2	86·9	86·5
	F_{2b}	59·3	100·0	100·0	12·4	99·5	92·9	68·5
	F_{3a}	65·2	100·0	100·0	10·7	96·3	90·9	62·3
	F_{3b}	78·9	100·0	100·0	12·5	100·0	82·4	81·2
200	F_{1a}	57·1	100·0	100·0	12·6	98·5	96·0	79·4
	F_{1b}	61·0	94·1	100·0	13·5	98·5	96·0	92·8
	F_{2a}	62·5	100·0	100·0	10·7	93·1	78·0	73·8
	F_{2b}	78·9	100·0	100·0	9·9	99·3	85·7	86·6
	F_{3a}	32·6	100·0	87·5	10·3	98·5	87·9	86·0
	F_{3b}	41·0	87·5	100·0	10·1	97·9	92·1	92·4
1000	F_{1a}	60·0	88·9	100·0	10·6	98·1	98·1	84·7
	F_{1b}	84·2	100·0	100·0	12·2	100·0	87·2	84·7
	F_{2a}	53·1	94·1	100·0	10·9	98·0	79·3	100·0
	F_{2b}	35·9	92·6	81·3	10·5	100·0	68·4	88·5
	F_{3a}	48·5	87·5	100·0	11·3	98·6	73·1	57·7
	F_{3b}	37·9	84·6	84·6	13·1	97·9	97·2	68·5

*Mating index = (copulations/oestrous cycles) × 100; fertility index = (pregnancies/copulations) × 100; pregnancy index = (pregnancies/females mated) × 100; live-birth index = (pups born viable/total pups delivered) × 100; 5-day survival index = (pups viable at day 5/pups born viable) × 100; lactation index = (pups viable at day 21/pups retained at day 5) × 100.

Table 5. *Effects of dietary levels of 0–1000 ppm compound IV on the reproductive indices of three generations of rats*

Dietary level (ppm)	Generation	Mating index*	Fertility index*	Pregnancy index*	Mean litter size	Live birth index*	5-Day survival index*	Lactation index*
0	F_{1a}	56·6	94·1	100·0	11·3	100·0	98·3	92·7
	F_{1b}	100·0	100·0	100·0	11·5	98·9	98·4	98·0
	F_{2a}	48·6	88·2	93·7	10·7	95·6	90·8	85·2
	F_{2b}	53·6	93·3	100·0	11·5	97·5	86·6	76·9
	F_{3a}	57·1	100·0	100·0	10·4	100·0	97·6	68·9
	F_{3b}	80·9	94·1	100·0	10·8	95·9	95·8	84·7
40	F_{1a}	59·2	100·0	100·0	12·9	99·0	94·1	93·2
	F_{1b}	72·7	100·0	100·0	12·7	98·0	76·9	82·8
	F_{2a}	55·2	100·0	100·0	9·9	96·2	66·0	96·9
	F_{2b}	72·7	100·0	100·0	11·2	93·3	92·8	66·9
	F_{3a}	46·9	93·3	93·3	12·0	100·0	78·6	61·1
	F_{3b}	61·5	88·0	100·0	12·1	99·4	91·1	81·5
200	F_{1a}	56·7	88·2	93·8	12·1	95·6	95·4	84·9
	F_{1b}	75·0	100·0	100·0	12·7	98·9	90·4	94·1
	F_{2a}	53·3	87·5	100·0	10·0	100·0	69·3	93·6
	F_{2b}	73·7	100·0	100·0	11·6	97·5	98·1	83·9
	F_{3a}	20·4	100·0	76·9	9·8	90·9	83·8	71·2
	F_{3b}	41·6	90·0	90·0	9·1	97·3	90·1	77·4
1000	F_{1a}	54·0	80·0	100·0	11·5	97·3	92·1	86·9
	F_{1b}	72·7	100·0	100·0	11·5	91·3	89·3	95·1
	F_{2a}	65·4	94·1	100·0	10·4	99·4	91·5	60·3
	F_{2b}	63·0	94·1	100·0	11·6	97·3	91·7	65·0
	F_{3a}	40·0	81·3	92·9	10·8	95·3	90·2	59·8
	F_{3b}	86·6	100·0	100·0	10·3	94·8	92·9	76·5

*Mating index = (copulations/oestrous cycles) × 100; fertility index = (pregnancies/copulations) × 100; pregnancy index = (pregnancies/females mated) × 100; live-birth index = (pups born viable/total pups delivered) × 100; 5-day survival index = (pups viable at day 5/pups born viable) × 100; lactation index = (pups viable at day 21/pups retained at day 5) × 100.

tissue from some of the dogs given 1000 ppm indicated an increase in haematopoiesis. All other tissues and organs were normal.

Compounds II, III and IV at dietary levels up to 2000 ppm exerted no adverse effects upon body weights, food consumption, survival, behaviour, haematology, clinical parameters, urine analysis or gross and microscopic pathology.

Three-generation reproduction study

Offspring from three consecutive generations of albino rats fed the fluorescent whitening agents at 40, 200 or 1000 ppm in the diet were free of gross external anomalies. The progeny exhibited normal growth and behaviour through lactation and the subsequent maturation period before producing the next generation. Gross and histopathological examination of parental animals killed in each generation revealed no adverse findings. Body weights, organ-to-body weight ratios, organ weights and organ-to-brain weight ratios in treated and control animals showed no differences that could be correlated with the ingestion of the test materials.

The effects of the FWAs on reproduction are summarized in Tables 2–5. With the exception of compound III, no alterations in the reproductive performance of the animals were observed. Animals fed 1000 ppm compound III (Table 4) showed a slight reduction in mating indices and incidences of pregnancy in the second litters of both the second and third generations (F_{2b} and F_{3b} litters). This finding was not considered significant since other reproductive parameters were normal. Survival of the resulting young was not adversely affected. Progeny survival indices calculated at various stages of lactation revealed no consistent dose- or generation-related reduction of pup survival attributable to the ingestion of any test material.

Gross and histopathological examination of randomly selected F_{3b} weanlings showed no differences between test and control progeny.

DISCUSSION

Numerous organic chemicals have been proposed for use as fluorescent whiteners and the chemical, physical and technological aspects of FWAs have been the subject of several reviews (Sakar, 1971; Stensby, 1967 & 1968). Since FWAs are intended for use in consumer products such as laundry detergents, the potential toxicity of these agents is of paramount importance. In general, the FWAs have a low order of acute, subacute and long-term toxicity (Gloxhuber, 1972; Gloxhuber, Hecht & Kimmerle, 1962; Snyder, Opdyke & Rubenkoenig, 1963) and are non-hazardous in terms of sensitization and photosensitization (Glashoff, Stegmann & Schroeder, 1963; Griffith, 1973; Herzberg, 1964; Schneider & Mirus, 1955; Snyder *et al.* 1963; Vonkennel, Schutz & Wulf, 1957).

With respect to the four FWAs included in the present study, Keplinger *et al.* (1974) reported that

the acute oral LD_{50} of each material in both adult mice and rats was greater than 5000 mg/kg. The acute oral LD_{50} in newborn rats was approximately 800, 933, 654 and 235 mg/kg for compounds I, II, III and IV, respectively. Keplinger *et al.* (1974) further noted that the four compounds had a low order of acute inhalation toxicity (2900–5500 mg/m^3) in adult rats and caused neither skin irritation nor sensitization in man.

Long-term and subacute oral toxicity studies with the four FWAs revealed similar results. No toxic effects were observed in rats given the materials at dietary levels up to 5000 ppm for 90 days or 1000 ppm for 2 yr. Compounds II, III and IV were not toxic to dogs at dietary levels up to 10,000 ppm for 90 days or 2000 ppm for 2 yr.

Dose-related effects ranging from mild gastritis at 400 ppm to debilitation and peritonitis at 2000 ppm were noted in dogs fed unbuffered compound I in the 90-day study. The findings were apparently related to the alkalinity of compound I, as repetition of the study using 2000 ppm compound I neutralized to pH 7 with hydrochloric acid showed no such effects. In the 2-yr study, a similar fibrous peritonitis was found in some of the dogs given 400 or 1000 ppm compound I. This finding represents the healing stage of an inflammatory process and was apparently due to the compound's high alkalinity (pH 10·5), which in turn was due to residual caustic soda from the manufacturing process. The study was at wk 30 when the high pH was discovered and thereafter compound I buffered to a pH of 7 was fed. Compounds II, III and IV, manufactured by a different process, had a pH of approximately 7.

Toxicological interest in the mode of action of FWAs, namely their ability to absorb ultraviolet radiation with subsequent emission of visible light, has led to investigations of their photobiological effects. Bingham & Falk (1970) reported that each of three FWAs tested (one coumarin derivative and two diaminostilbene disulphonate derivatives) increased the incidence of skin tumours in mice when applied by skin-painting and exposed to irradiation with ultraviolet light (60% of the energy at 254 nm). The study was considered to be unrealistic, however, in that the wavelength of light used is not present in the sunlight reaching the surface of the earth. Since the FWAs studied in the present investigation are used commercially, the potential photocarcinogenicity of two of the compounds was examined by Forbes & Urbach (1973) under more realistic experimental conditions. They found that compounds I and III did not enhance photocarcinogenesis when applied topically to mice which were then irradiated at 300–1000 nm using solar simulators. Under similar experimental conditions, the known phototoxic agent 8-methoxypsoralen enhanced light-induced carcinogenesis. Forbes & Urbach (1973) further reported that compounds I, II, III and IV did not induce phototoxicity or photosensitivity when tested on the skin of human subjects, hairless mice and miniature swine.

The potential carcinogenicity of compounds I and III has been examined by other investigators. Neukomm & De Trey (1961) showed that compounds I and III were not carcinogenic when administered orally or sc to mice, and Snyder *et al.* (1963) found that compound I did not induce skin tumors in mice.

Mutagenicity of the four FWAs has been investigated by Keplinger *et al.* (1974). None of the compounds produced a mutagenic effect in mouse germinal cells when tested in a dominant lethal assay. It is noteworthy that interest in the possible mutagenic effects of FWAs has arisen from the work of Gillberg & Åman (1971), who reported that certain FWAs produced petite mutants in a diploid yeast strain both in the presence and absence of light. However, Leuthold (1972) failed to confirm these results and Kilbey & Zetterberg (1973) subsequently showed that the effects obtained by Gillberg & Åman (1971) could be attributed to the selection of pre-existing mutants under the conditions used. Moreover, Gillberg is reported to have stated, at a Swedish symposium on FWAs, that he cannot now obtain positive results with the compounds that he formerly tested (Kilbey & Zetterberg, 1974). Kilbey & Zetterberg (1973) also found that nuclear gene changes did not occur upon exposure of the yeast to the FWAs, and noted that the petite mutation induction test has doubtful value as an indicator of potential nuclear mutagens. Consequently, mammalian *in vivo* test systems appear to have more relevance when assessing the potential mutagenic hazard of FWAs to man.

The reproductive effects of the four FWAs were studied in the present investigation. None of the compounds had an adverse effect on mating, fertility or pregnancy indices when fed to male and female albino rats throughout a three-generation reproduction study at dietary levels up to 1000 ppm. Survival and lactation indices of the newborn pups did not deviate from the normal, and no teratogenic effects were observed in the pups. Similarly the four FWAs were not teratogenic for rabbits (Keplinger *et al.* 1974). The low toxicity of the agents was established further by the finding that the materials did not cause any adverse gross or histopathological changes in the parental rats or progeny.

In conclusion, the four FWAs reported in this paper have been extensively evaluated in both acute and long-term toxicity studies. Even after ingestion for 2 yr, tissue levels of the FWAs were either not detectable or were extremely low (0·63 ppm or less). The results of this study, as well as of the investigations cited, indicate that the use of commercial products containing any of these four compounds poses a minimal hazard to the consumer.

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LONG-TERM SAFETY STUDIES OF A CHLOROFORM-CONTAINING DENTIFRICE AND MOUTH-RINSE IN MAN

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Abstract—The safety of a dentifrice containing 3·4% chloroform and a mouth-wash containing 0·43% was assessed in two long-term usage studies lasting for 1 and 5 yr and involving 229 subjects. The safety of the preparations was monitored by measuring liver function, using tests for serum glutamic–pyruvic and glutamic–oxalacetic transaminases, serum alkaline phosphatase and blood urea nitrogen. The results showed that for these parameters there were no statistical differences between experimental and control subjects.

INTRODUCTION

Chloroform has been formulated in pharmaceutical and toiletry products for many years and its toxicity has been investigated extensively (Challen, Hickish & Bedford, 1958; Plaa, Evans & Hine, 1958; Scholler, 1968; Wallace, 1950; Zimmerman, 1968). The major toxic effect is liver damage, which is reflected in raised serum levels of glutamic–pyruvic and/or glutamic–oxalacetic transaminases (SGPT and SGOT; Bombeck, Aoki, Smuckler & Nyhus, 1969; Cornish & Block, 1960; Ramsöe, Tygstrup & Winkel, 1970; Thorpe, Gopinath, Jones & Ford, 1969; Wirtschafter & Tsujimura, 1961; Wroblewski, Jervis & LaDue, 1956) and alkaline phosphatases (SAP; Bombeck *et al.* 1969; Thorpe *et al.* 1969) and in changes in serum triglyceride (Zimmerman, 1968).

The safety of the long-term usage of a mouth-wash containing 0·43% chloroform and a dentifrice containing 3·4% has now been investigated in a two-part programme involving 229 subjects. One study was conducted for 12 months using the daily combination of mouth-wash and dentifrice and the other for 60 months with the daily use of the dentifrice. In these studies, the parameters monitored were SGPT, SGOT, SAP and blood urea nitrogen (BUN). Clinical observations and blood-chemistry data produced no evidence that any adverse changes were induced. Details of these findings are the subject of this report.

EXPERIMENTAL

The studies were conducted at two state hospitals for mentally retarded and/or physically handicapped residents. All the participants were ambulatory and performed the task required under proper supervision. The age and sex distributions for the various groups in both studies are shown in Table 1. The records did not include information on the age and/or sex of five subjects in study I or on the age of twelve subjects and the sex of five in study II.

Medication of the residents continued throughout the investigation; most subjects received at least one drug, while many received several. The following types of medication were administered concurrently during the two studies: Hypnotic—flurazepam (Dalmane); haematinic—ferrous sulphate; anticonvulsants—primidone (Mysoline) and diphenylhydantoin (Dilantin); antibiotics—penicillin and tetracycline; antibacterial—sulphadiazine; antihistamines—tripelennamine (Pyribenzamine) and diphenhydramine (Benadryl); antihistamine–decongestant combination—brompheniramine/phenylephrine/phenylpropanolamine (Dimetapp); antihistamine–analgesic combination—chlorpheniramine/aspirin/caffeine (Coricidin); diuretic—hydrochlorothiazide (Hydrodiuril); diagnostic aid—sodium dehydrocholate (Decholin); antiarrhythmic—propanolol (Inderal); hormonal formulation—testosterone cypionate/oestradiol cypionate (Depo-testadiol); anticholinergics—benzotropine (Congentin) and bioperidin (Akineton); hypoglycaemic—insulin; laxative—bisacodyl (Dulcolax); tranquillizers—fluphenazine (Prolixin), trifluprom azine (Vesprin), chlorpromazine (Thorazine), haloperidol (Haldol), trifluoperazine (Stelazine) and chlorprothixene (Taractan); minor tranquillizers—chlordiazepoxide (Librium), diazepam (Valium) and oxazepam (Serax); antidepressants—thioridazine (Mellaril), doxepin (Sinequan), nortriptyline (Aventyl) and imipramine (Tofranil); stimulants—*d*-amphetamine (Dexedrine) and methyl phenidate (Ritalin); urinary analgesic—ethoxazene (Serenium); urinary antiseptic—meth-

Table 1. *Age and sex distributions of subjects participating in studies I and II*

Group	No. of subjects in age group (yr)					No. of		Total no.
	20	21–30	31–40	41–50	50	Males	Females	
Study I (60 months)								
Control	14	17	15	5	6	20	37	59*
Treated	20	15	12	7	3	21	35	59†
Total...	34	32	27	12	9	41	72	118
Study II (12 months)								
Control	0	3	6	14	24	24	27	54‡
Treated	0	6	11	17	18	34	21	57§
Total...	0	9	17	31	42	58	48	111

*Age and sex of two subjects not recorded.
†Age of two subjects and sex of three not recorded.
‡Age of seven subjects and sex of three not recorded.
§Age of five subjects and sex of two not recorded.

Table 2. *Summary of serum transaminase values redorded in study I*

Duration of study (months)	SGOT* (Frankel units)		SGPT* (Frankel units)	
	Controls	Treated group	Controls	Treated group
12	10·0 ± 5·9 (59)	8·4 ± 7·0 (59)	8·9 ± 8·1 (59)	7·7 ± 5·6 (59)
18	17·4 ± 9·3 (59)	17·6 ± 1·0 (59)	15·1 ± 9·9 (54)	15·6 ± 8·9 (59)
24	14·0 ± 4·6 (59)	14·6 ± 6·9 (59)	10·8 ± 7·6 (59)	9·4 ± 7·5 (59)
30	16·8 ± 18·2 (59)	15·2 ± 2·1 (59)	15·5 ± 7·3 (59)	13·1 ± 5·6 (59)
36	10·2 ± 5·5 (58)	9·6 ± 5·1 (57)	4·4 ± 4·0 (58)	4·3 ± 5·3 (57)
42	8·8 ± 6·2 (57)	9·2 ± 6·0 (57)	6·6 ± 7·0 (57)	5·9 ± 6·3 (57)
48	6·8 ± 4·6 (56)	6·9 ± 3·6 (57)	4·8 ± 3·8 (56)	5·1 ± 5·0 (56)
54	11·2 ± 4·2 (53)	11·4 ± 6·1 (51)	9·1 ± 5·1 (53)	9·8 ± 9·6 (52)
60	10·5 ± 5·0 (51)	11·7 ± 8·3 (50)	7·3 ± 4·9 (51)	10·7 ± 16·2 (50)
Overall mean...	11·8 (511)	11·7 (508)	9·2 (506)	9·1 (508)

SGOT = Serum glutamic–oxalacetic transaminase SGPT = Serum glutamic–pyruvic transaminase
*Values are expressed as the means ± SD for the number of determinations indicated in parenthesis.

enamine (Delamine-500); depressant—phenobarbitone; antiparkinsons—trihexphenidyl (Artane); vitamine B_{12}.

Study I. The subjects were 118 men and women assigned for 60 months to two groups of equal size. One group (treatment group) used a dentifrice containing 3·4% chloroform for brushing the teeth, while the other (control) group used a non-chloroform dentifrice. Each subject brushed his teeth for 1 min twice daily, using about 1 g of the respective toothpaste each time. The brushing of teeth was supervised, and intra-oral clinical evaluation was carried out before and at 3-monthly intervals during treatment. The monitoring of SGPT and SGOT was initiated 6 months after the start of the dental evaluation programme and was repeated periodically. SAP and BUN measurements were made randomly for about 1 yr after the beginning of the dental programme.

Study II. The 111 men and women involved were assigned to two groups for 1 yr. One group of 57 (treatment group) brushed their teeth with the 3·4% chloroform-containing dentifrice and used a 0·425% chloroform-containing mouth-rinse, while a group of 54 (control group) used a non-chloroform dentifrice and mouth-rinse. Dentifrice (1 g) and mouth-rinse (15 ml) were used twice daily by all subjects, under supervision. Intra-oral clinical evaluations were carried out before and at 3-monthly intervals during the study. Transaminase levels were determined before the study and at months 6, 9–10 and 12.

Analytical methods. SGPT and SGOT assays were generally done manually by the method of Reitman & Frankel (1957). At and after month 20 of study I, however, SGOT was measured with SMA 12/60 (Beckman Instrument Co., Mountainside, N.J.), as a diazonium salt, and SGPT with a Harleco kit (Harleco, Gibbstown, N.J.). BUN and SAP were measured with SMA 12/60. The normal ranges for these determinations are 8–40 and 5–30 Frankel units for SGOT and SGPT, respectively, 10–30 mg/100 ml for BUN and 3–85 IU for SAP.

RESULTS

Study I

The data for transaminase and other liver-function tests are summarized in Tables 2 and 3, respectively. Of the original 118 subjects, 103 completed the 60-month regimen. Seven control and eight treatment-group patients were dropped for reasons unrelated to the use of the dentifrice. The 30-month data was analysed statistically because it included transaminase measurements for all 118 subjects. Since normal serum-transaminase values tend to fluctuate (e.g. SGOT 8–40 and SGPT 5–30 units/ml), values that exceeded the upper limits were treated as abnormal and those within or below the normal range as not differing significantly from normal.

For SGOT, the treated and control groups each contained 57 normal and two abnormal values at

Table 3. *Summary of SAP and BUN values recorded in study I*

Duration of study (months)	SAP* (IU)		BUN* (mg/100 ml)	
	Controls	Treated group	Controls	Treated group
12	5·0 ± 0·6 (5)	4·7 ± 0·6 (10)	23·2 ± 2·5 (4)	22·6 ± 1·3 (5)
18	4·7 ± 0·3 (8)	4·9 ± 0·5 (10)	22·0 ± — (1)	23·0 ± — (1)
24	4·6 ± 0·4 (9)	5·1 ± 0·7 (8)	24·0 ± 1·4 (4)	24·7 ± 4·6 (3)
36	4·8 ± — (1)	4·5 ± 0·3 (4)	30·0 ± — (1)	22·0 ± 0·0 (3)
48	4·4 ± 0·1 (3)	4·7 ± 0·6 (6)	22·0 ± — (1)	
Overall mean...	4·7 (26)	4·8 (38)	23·9 (11)	23·0 (12)

SAP = Serum alkaline phosphate BUN = Blood urea nitrogen
*Values are expressed as means ± SD for the number of determinations indicated in parenthesis.

Table 4. *Summary of serum transaminase values recorded in study II*

Duration of study (months)	SGOT* (Frankel units)		SGPT* (Frankel units)	
	Controls	Treated group	Controls	Treated group
0†	21·1 ± 18·7 (52)	17·4 ± 6·2 (57)	11·7 ± 10·1 (52)	8·4 ± 8·2 (57)
6	25·4 ± 13·2 (52)	27·7 ± 18·8 (62)	10·6 ± 9·1 (52)	9·8 ± 9·2 (61)
9–10	22·4 ± 15·5 (51)	21·4 ± 13·0 (51)	14·9 ± 8·9 (50)	14·2 ± 8·3 (51)
12	20·2 ± 10·3 (50)	20·5 ± 9·0 (52)	18·0 ± 6·8 (49)	17·8 ± 5·9 (52)
Overall mean...	22·3 (205)	21·9 (222)	13·7 (203)	12·3 (221)

SGOT = Serum glutamic–oxalacetic transaminase SGPT = Serum glutamic–pyruvic transaminase
*Values are expressed as the means ± SD for the number of determinations indicated in parenthesis.
†Pretreatment value.

month 30 ($\chi^2 = 0{\cdot}0001$), while for SGPT, there were 54 normal and five abnormal values in the treated group and 55 normal and four abnormal in the controls ($\chi^2 = 0{\cdot}1209$). Thus chi-squared tests for SGOT and SGPT showed that there were no differences between treated and control values. Examination for double dichotomy showed four and nine abnormal results, respectively, for SGOT and SGPT in the 118 subjects. The probability of every split of these abnormal subjects was calculated with a computer and the significance of a given split was the sum of individual probability of the observed plus the probability of each more extreme split to the ultimate, in this case 0 and 4 and 0 and 9, respectively. Iterations 1–6 showed total P to be 0·69 for SGOT and 0·75 for SGPT, demonstrating no differences between control and treated groups.

The corresponding relationships between transaminase values for the control and treated groups at month 60 were similar, the 101 subjects considered at this time giving 49 normal SGOT values and one abnormal one in the treated group while all 51 in the control group were normal ($\chi^2 = 1{\cdot}0202$), and 48 normal and two abnormal SGPT values in the treated group with all 51 in the control group again normal ($\chi^2 = 2{\cdot}0612$). SAP and BUN values (Table 3) showed no differences between control and treated groups.

Study II

The transaminase data for this study is summarized in Table 4. The SGOT values were normal in 44 and abnormal in 11 of the treated group and normal in 41 and abnormal in ten of the control group ($\chi^2 = 0{\cdot}0025$), while the SGPT values were 54 normal and one abnormal for the treated group and 49 normal and two abnormal for the controls ($\chi^2 = 0{\cdot}4305$).

When two-by-two contingency tables are constructed from these figures and the tables are considered as double dichotomies, the exact probabilities of observed splits computed by hypergeometric methods are 0·61 for SGOT and 0·47 for SGPT. Each is almost an even split, demonstrating no difference between treated and control subjects for both tests.

DISCUSSION

It is recognized (Zimmerman, 1968) that hepatotoxicity is an adverse effect of excessive amounts of chloroform, and a correlation between liver damage and elevated blood-transaminase values has been reported (Wirtschafter & Tsujimura, 1961). Because of the published toxic effects, there is the possibility of misconception that chloroform at any level is unsafe for prolonged usage. This investigation was conducted to demonstrate the safety of chloroform in a mouth-wash and in a dentifrice. In the present studies, many of the subjects, during their confinement, were taking drugs that could adversely affect liver function. The uniqueness of the situation presented an opportunity to evaluate the question of long-term safety.

In study I, a total of 68 mg chloroform was in the toothpaste used daily; in study II, the dentifrice and mouth-rinse used daily contained 68 and 129 mg chloroform respectively. If it is assumed that all the chloroform was ingested, the total intake for a 50-kg adult in study I was 1·36 mg/kg and in study II 3·94 mg/kg; for a 3- or 4-yr-old child weighing 20 kg it was 3·4 mg/kg in study I and 9·85 mg/kg in study II. It was reported by Hargreaves, Ingram & Wagg (1970) that children ingest on average less than 25% of the dentifrice during normal brushing. Assuming that the mean ingestion of dentifrice is 25% for both adult and child, the total body burden can be recalculated. In study I the adult exposure is 0·34 mg/kg and in study II 0·96 mg/kg; the child exposure for study I is 0·85 mg/kg and for study II 2·46 mg/kg.

Wallace (1950) reported that daily ingestion of 1·6–2·6 g chloroform with 0·8–1·3 g codeine daily for 10 yr produced hepatotoxicity, which was reversible. In rats, 2·5 g chloroform/kg ip caused liver damage (Brauer & Root, 1946). Klaassen & Plaa (1969) compared the biochemical changes in the livers of rats and found that 1·3 ml chloroform/kg ip induced only a slight depression of glucose-6-phosphatase. Our own experience of monkeys receiving 100 mg/kg and rats receiving 55 mg/kg by gavage for 1 yr showed no systemic toxicity associated with chloroform administration (data to be published). Therefore, in view of the toxicological data cited and the amount of chloroform in the dentifrice and mouthwash, the safety margin is considerable. To repeat this study with young children is not warranted, since it is possible to make adequate extrapolation from the data obtained from juveniles and adults to show safety in young children.

Originally, study I was designed to assess the effect of the dentifrice on oral tissue. The tissue was not adversely affected and the results are to be reported in more detail elsewhere (Volpe, Mooney, Zumbrunnen, Stahl & Goldman, 1975). It became evident

during the first 6 months that a concomitant appraisal of liver function should also be included. When study II was planned, the safety aspect was an integral part of the design. It was scheduled for 1 yr with baseline pretreatment measurements of transaminase values. This 1 yr was considered sufficient, since observation for 2 yr had already been accumulated from study I and the total chloroform load was almost three times that used in study I.

Statistical analysis of the transaminase values in both studies showed that there were no differences between the subjects using chloroform-free and those using chloroform dentifrices. The monitoring of control and experimental subjects represents a unique collection of transaminase data for a large population over prolonged periods of time. The parallel between the values for control and experimental subjects indicates normal fluctuation within the population. Certainly, when considering the range accepted as normal for these tests, the observations are in good accord. In fact, the treatment of the data statistically as nonparametric demonstrates this. There are several ways in which the data could be analysed statistically. We have tabulated the data as means with standard deviations and it is obvious on inspection that there are no differences between treatment and control groups. To stress the data, we questioned whether or not there were deviations above the normal physiological ranges and performed the chi-squared evaluations. These confirmed the lack of differences between treatment and control groups.

Transaminase values, especially in the long term, are a good index of hepatic vitality. The absence of differences supports the thesis that chloroform at 3·4% in dentifrice and 0·43% in a mouth-rinse is not a medical hazard, even to individuals who may be using medication with some potential for inducing liver dysfunction.

Van Dyke, Chenoweth & Van Poznak (1964) used material labelled with ^{14}C and ^{36}Cl to show that, in rats, some 4·5% of a dose of chloroform given ip was eliminated as $^{14}CO_2$ in the exhaled air and up to 2% as an inorganic salt in the urine. Following ingestion of ^{13}C-labelled chloroform in mineral oil by human adults (Fry, Taylor & Hathway, 1972), up to 70% of the label was recovered as unchanged chloroform in the exhaled air and up to 50% as $^{13}CO_2$, less than 0·01% of the chloroform being excreted unchanged in the 8-hr urine. The studies show that when chloroform is deliberately swallowed, an appreciable amount is exhaled unchanged and a smaller amount is absorbed systemically.

In our studies, it would have been difficult to quantify individually how much chloroform was absorbed. It is reasonable to assume that a certain amount was, but this amount, on a long-term basis (1–5 yr), did not induce a measurable change in liver function. The findings support our judgement that chloroform in a dentifrice and/or a mouth-rinse is not a medical hazard. Furthermore, they illustrate that safety should be judged in terms of hazard, with toxicity *per se* being no indictment against safety.

Any substance, be it a cosmetic, preservative, flavouring or drug, for example, has toxicological attributes, especially when used in very high concentrations and under exaggerated conditions of use. What must be ascertained is whether or not this toxic potential is inducible during reasonable or anticipated usage. We have directed our efforts to this end, and the results of our studies substantiate the safety of chloroform in a dentifrice and/or mouth-rinse.

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THE RABBIT AS A MODEL FOR EVALUATING SKIN IRRITANTS: A COMPARISON OF RESULTS OBTAINED ON ANIMALS AND MAN USING REPEATED SKIN EXPOSURES

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Abstract—A new animal test for evaluating the skin-irritant capacity of cosmetic and drug preparations and ingredients intended for repeated application is presented. The test is patterned on the 21-day occlusive test on man. Substances are applied 16 times (uncovered) to the skin of rabbits over a 3-wk period. The two parameters of the inflammatory response evaluated (16 times) throughout the test as indices of skin irritation are redness (cumulative irritation score) and fluid accumulation (changes in skinfold thickness). Measurements of these two parameters are highly correlated ($r = 0{\cdot}86$; $P < 0{\cdot}001$). Results on 60 test materials showed a significant correlation ($r = 0{\cdot}30$; $P < 0{\cdot}02$) between the cumulative irritation scores obtained on rabbits and those obtained on man. The usefulness and limitations of single-application (Draize type) and multiple-application tests for skin irritation are discussed. Exploratory results with hairless rats and hairless hamsters suggested that these species were not sufficiently responsive to be useful in skin-irritation studies. A radioimmunoassay of skin prostaglandin E disclosed no differences between irritated and control skin, suggesting that this technique cannot be used as an objective measure of skin irritation.

INTRODUCTION

Substances that are applied to human skin may be readily tolerated, producing neither subjective nor objective evidence of adverse effects, or they may produce itching and/or gross or microscopic skin lesions on single or repeated application (irritant-contact dermatitis). This paper is concerned with primary irritant-contact dermatitis. This results from direct cytotoxicity, in contrast to allergic contact dermatitis (delayed skin hypersensitivity), which has an immunological basis. This paper presents a new animal test for evaluating skin irritants and compares the data obtained by this method with data from a 21-day occlusive test conducted in man (Phillips, Steinberg, Maibach & Akers, 1972). The latter test is a modification of earlier methods (Finkelstein, Laden & Miechowski, 1963; Kligman & Woodring, 1967; Lanman, Elvers & Howard, 1968).

Cellular injury from topically applied substances is characterized by a local inflammatory response, manifested by two important macroscopically visible events, a reddening of the skin (erythema) and accumulation of fluid (oedema). By observing or measuring these changes one can estimate the extent of skin damage that has occurred.

Skin-irritation tests have been conducted on guinea-pigs (Finkelstein *et al.* 1963; Roudabush, Terhaar, Fassett & Dziuba, 1965), rabbits (Finkelstein *et al.* 1963), rats (Brown, 1971; Finkelstein *et al.* 1963) and mice (Schmid, 1970), as well as man. The majority of these tests involved a single application of test material under occlusion, after which skin reactions were scored at 24 and sometimes 72 hr. Often the procedure was some modification of the Draize technique (Draize, Woodard & Calvery, 1944). In only two instances were results of such tests compared with results of simultaneous tests on man (Brown, 1971; Phillips *et al.* 1972); their usefulness in predicting the human response is therefore unknown. One study involving surfactants tested on human and animal skin (Brown, 1971) concluded that "a cautious approach to predictive skin testing with detergent is advocated" and "predictive testing in animals is at least as useful as comparable tests on humans".

Since topical drug preparations are applied for days or even weeks, and cosmetics may be applied for a lifetime, it was felt that a reasonable approach to establishing the safety of preparations intended for prolonged use is an animal test based on repeated application. Not only does multiple application more closely duplicate conditions of use; it also provides the opportunity for making repeated observations of the skin. When conducted over a 3-wk period, the use of multiple scorings obviously adds precision to the evaluation and should increase the capacity for discrimination between related final formulations of relatively low irritancy. It was decided to develop a new animal test for evaluating the irritancy of applied chemical substances on the basis of the 21-day human occlusive patch test.

Hairless animals have increased corneal thickness and their skin-permeability characteristics more closely approximate those of man than do those of the haired varieties (Stoughton, 1974). Accordingly, exploratory studies were conducted on hairless rats and hairless hamsters. Unfortunately these varieties showed little capacity to respond to skin irritants (F. N. Marzulli & H. I. Maibach, unpublished data 1974), and it was therefore decided to use albino rabbits in the present work. The test material was applied

daily to the uncovered clipped skin, and results obtained in a 16-day animal test were compared with those obtained in the 21-day test on occluded human skin. The latter test, described previously by Phillips *et al.* (1972), appears to be useful for characterizing the irritant potential of topical preparations that may be used on man. Uncovered rabbit skin was used because rabbit skin is some four or more times as permeable as human skin when uncovered (Tregear, 1966), but is roughly comparable in permeability to occluded human skin, occlusion being thought to increase the permeability of human skin by about four or more times (Barr, 1962).

Irritation tests should be carried out on healthy intact skin. However, it is also important to know what happens when a mild irritant is applied to abraded or broken skin.

EXPERIMENTAL

Materials

The test substances consisted of ingredients of cosmetics or drugs and marketed preparations, samples of which were subdivided for use on both man and rabbits. The following materials were studied:

Group I: *Antimicrobials and preservatives.* Zinc pyrithione (Omadine®, Olin Mathieson Chemical Corp., Stamford, Conn.), sodium pyrithione, MDS pyrithione (magnesium adduct) and hexachlorophene, each in three concentrations.

Group II: *Sunscreens.* The active ingredients, *p*-aminobenzoic acid (PABA), homomenthyl salicylate (homosalate), sulisobenzone (5-benzoyl-2-methoxybenzene sulphonic acid; Uvinul MS-40®, GAF Corp., New York), 2-ethoxyethyl *p*-methoxycinnamate (Giv Tan F®, Givaudan Corp., Clifton, N.J.) and menthyl anthranilate, each in two concentrations, and the marketed sunscreen preparations, Beach Party Sun Tan Lotion®, containing 2-ethoxyethyl *p*-methoxycinnamate, coconut oil, cocoa butter and Vitamins A, D and E (Walgreen Laboratories, Inc., Chicago, Ill.) and Coppertone®, containing amyl *p*-dimethylaminobenzoate (Escalol 506®, Van Dyke, Inc., Belleville, N.J.), homosalate, propylene glycol, methylparaben and triethanolamine (Plough Inc., Memphis, Tenn.).

Group III: *Acids and alkalis.* Salicylic acid, oxalic acid and sodium hydroxide in three or five concentrations.

Group IV: *Detergents.* Sodium lauryl sulphate in four concentrations and a bubble bath product (Mr. Bubble®, Gold Seal Co., Bismark, N.D.) in five concentrations. Analysis (H. M. Davis, Division of Cosmetics, Food and Drug Administration) indicated that the latter contained 8·3% sulphated surfactants, including sulphonated amide and possibly olefin sulphonate.

Group V: *Antiperspirants.* The active ingredient, aluminium chlorhydroxide complex, and the marketed preparations, Right Guard Super Dry® containing aluminium chlorhydroxide complex (Gillette Co., Boston, Mass.) and Sure® containing aluminium chlorhydroxide-zirconium chlorhydroxide-glycine complex (Procter & Gamble Co., Cincinnati, Ohio).

Group VI: *Vitamin E (marketed preparations).* Mennen E Deodorant® containing 2% *dl*-tocopherol (Mennen Co., Morristown, N.J.), Natural E-Cream containing 12% vitamin E (form?) in wheat germ, 100 IU/g (Korvettes Products, Inc., New York) and Natural Vitamin E Oil containing 32% mixed tocopherols in a natural base of wheat germ and vegetable oils, 24,000 IU/2 oz (Korvettes Products, Inc.).

Group VII: *Cosmetics ingredients.* Ethyl alcohol, mineral oil, petrolatum and deodorized kerosene in one or three concentrations.

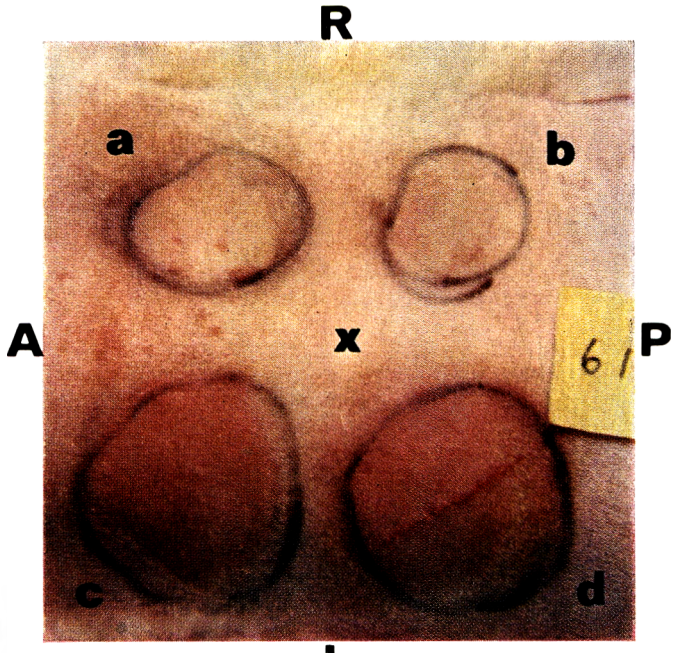
Group VIII: *Marketed cosmetic skin preparations.* Ponds Cold Cream® containing mineral oil (Chesebrough-Pond's Inc., Clinton, Conn.), Vaseline Intensive Care® (Chesebrough-Pond's Inc.), Noxzema® containing phenol, camphor, clove oil, eucalyptus and menthol (Noxell Corp., Baltimore, Md) and Ultra Sheer Make Up Moisturizer® (UK).

Group IX: *Antipsoriatic drug.* Anthralin, in two concentrations.

Methods

16-Day cumulative irritation test in rabbits (uncovered test site). Six New Zealand albino rabbits were used for each test. The animals' backs were clipped on a Saturday, and on two subsequent Saturdays. Each test material was applied 14 times, on Mondays to Fridays except for the last Friday. About 0·05 ml of the test material was applied to one of four sites on the back of each clipped rabbit (Fig. 1), two anterior and two posterior, two being on each side of the dorsal midline. Each test site was scored visually, together with one centrally located control site, and some were measured for skinfold thickness to the nearest 0·1 mm (Lange skinfold thickness caliper, Cambridge Scientific Industries, Cambridge, Md; Fig. 2) at 24 hr and every subsequent day except Sundays (16 readings). The scores used were 0 for no erythema, 1 for barely perceptible erythema, 2 for well-defined erythema, 3 for moderate to severe erythema and 4 for beet-red erythema to slight eschar and/or disruption of epidermal intactness. Scaliness was arbitrarily assigned a score of 1. At the conclusion of the test, the cumulative score for each animal (maximum $16 \times 4 = 64$) was obtained and the mean cumulative score for six rabbits was recorded as an index of skin irritation. Change in skinfold thickness, primarily a measure of fluid accumulation in the skin, was evaluated by deriving the mean difference from the daily differences between test and control-site readings on each rabbit and using the average of these mean differences for six rabbits as an index of skin irritation. The procedure, it should be noted, is somewhat different from that used for obtaining a cumulative score.

21-Day cumulative irritation test in man (covered test site). Six adult white test subjects were used (Phillips *et al.* 1972). Each test material (about 0·05 ml) was applied to the backs of the subjects under occlusion (Non-woven Webril® pad (Kendall Co. Boston, Mass.) with acrylic tape, Blenderm® (Minnesota Mining Co., St. Paul, Minn.). Each patch was removed at 24 hr. The skin was scored 30 min later and new material and a fresh patch were applied. The scores used were 0 for no erythema, ± for questionable erythema, 1 for well-defined erythema, 2 for erythema and induration, 3 for vesiculation and 4 for a bullous reaction. As described for rabbits, the cumulative



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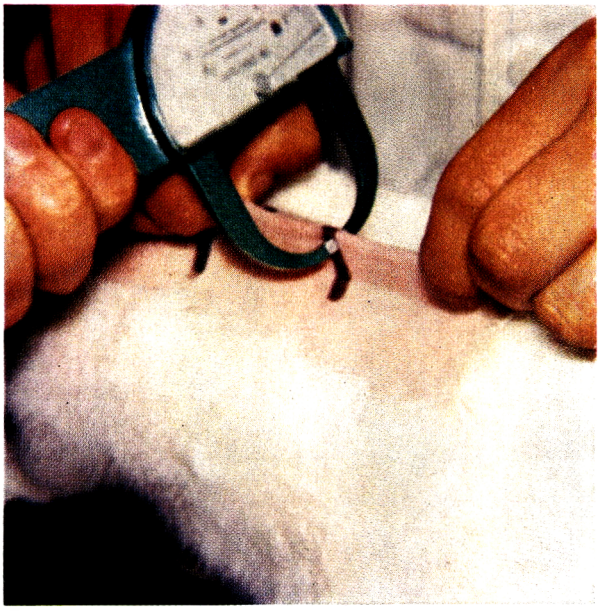

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Fig. 1. Rabbit back (A, anterior; P, posterior; R, right; L, left), showing a control site (X) and four sites of application of test materials: (a) Vitamin E oil, score 1; (b) Vitamin E cream, score 0; (c) Mennen E (intact skin), score 3; (d) Mennen E (abraded skin), score 3. Abrasion was accomplished with a scalpel. Effective, disposable plastics skin-abraders were also available from Maryland Plastics, 215 E. Central Avenue, Federalsburg, Md 21632.

Fig. 2. Use of the Lange caliper for measuring skinfold thickness to the nearest 0·1 mm. Skin should be measured at the same site each time, as close to the fold as possible. The caliper measures with a constant tension of 10 g/mm^2. Readings must be made within a few seconds to avoid compression of soft tissue. While any individual reading may be in error, errors tend to cancel when many readings are taken.

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score was obtained for each test material and the mean cumulative score for six subjects was used as an index of irritation. When a given site showed a bullous response (grade 4), no further applications were made and the site continued to receive a score of 4 for the remaining period. By contrast, in the rabbit test, application was continued even when severe skin damage occurred.

RESULTS

Results obtained with 11 substances tested on clipped rabbits (uncovered) by the 16-day cumulative irritation test are given in Table 1. The findings demonstrate a high correlation ($r = 0{\cdot}86$; $P < 0{\cdot}001$) between the two irritation parameters evaluated (redness and change in thickness of skin) using these materials. When a second control site (anterior to the first) was compared with the first control site, there was virtually no difference in skinfold thickness (+0·03 and +0·02; Table 1). This provides some measure of the reproducibility of the technique at a second skin site. A mean cumulative irritation score above 30 or a mean increase in skinfold thickness above 0·40 mm may serve as a warning that the test substance may be irritating when applied repeatedly to human skin.

The mean cumulative scores for sodium lauryl sulphate and for Sure (Table 1) were low in comparison with their scores for skinfold thickness, placing them out of order in the series. Apparently in some cases skin thickening may occur in the absence of a comparable increase in redness, if it involves mainly epidermal rather than dermal changes and is manifested primarily as an increase in cellular layers (acanthosis) rather than as fluid accumulation. This suggests that the mechanism by which irritants produce an inflammatory response may not always be the same.

Table 2 shows a comparison of cumulative irritation scores by the 21-day human and 16-day rabbit test using antimicrobial and preservative test materials (group I). In every instance the cumulative score for application to uncovered rabbits was greater than that obtained on man, indicating a greater responsiveness of rabbit skin to this type of material. With both test species, the cumulative irritation score increased with increasing concentrations of the active ingredient. The cumulative scores for both rabbits and man suggest that 3% hexachlorophene and 2% sodium pyrithione may produce skin irritation in some members of the population if applied repeatedly to the skin and left on. They also suggest that sodium pyrithione is more irritating than zinc or MDS pyrithione. The cumulative irritation scores obtained on rabbits show a significant correlation ($r = 0{\cdot}87$; $P > 0{\cdot}001$ but $< 0{\cdot}01$) with those obtained on man.

Table 2 also presents a comparison of cumulative irritation scores for man and rabbits using sunscreen ingredients and marketed preparations (group II). In ten of 12 tests, the rabbit showed a greater cumulative irritation score than man. One substance, sulisobenzone (Uvinul MS-40), appeared to be significantly more irritating to human skin than to rabbit skin. Although active ingredients were tested at two concentrations, an order of magnitude apart, the difference in results between concentrations was generally not impressive.

A comparison of cumulative irritation scores for man and rabbits using acids and alkalis (group III) is shown in Table 2. The scores for salicylic acid and sodium hydroxide are roughly comparable for man and rabbit, but oxalic acid appears to be somewhat more irritating to man than to rabbits. Table 3 gives results obtained with the materials and marketed preparations included in groups IV–IX. In three instances an active ingredient was tested with two different vehicles (Groups IV and V) and in one case a repeat test was run (Group VIII) to check the reproducibility of test results.

The results for all tests (60 values) were evaluated and cumulative irritation scores for man and rabbit showed a significant correlation ($r = 0{\cdot}30$; $P < 0{\cdot}02$ but $> 0{\cdot}01$) as indicated in Fig. 3.

The effects of three materials on abraded rabbit skin were compared with those on intact skin. In these cases the mean cumulative irritation scores on

Table 1. *Comparison of two skin-irritation parameters (skinfold thickness and mean cumulative irritation score) in clipped albino rabbits tested for 16 days with 11 different materials*

Compound or test material	Vehicle	Skinfold thickness* (change from control)	Mean cumulative score*
Sure†	—	+0·95	31·3
Anthralin (0·1%)	P	+0·79	56·0
Hexachlorophene (3·0%)	P	+0·58	44·1
Sodium lauryl sulphate (1·0%)	P	+0·34	6·5
Vaseline Intensive Care	—	+0·31	26·5
Deodorized kerosene	—	+0·31	17·1
Noxzema	—	+0·22	11·8
Sulisobenzone (1·0%)	A	+0·17	3·6
Oxalic acid (1·0%)	P	+0·13	3·5
Bubblebath (10%; Mr. Bubble)	W	+0·14	2·5
PABA (10%)	P	+0·05	2·5
Control site 2	—	+0·03	0·0
Control site 2′	—	+0·02	0·0

P = Petrolatum W = Water A = Alcohol (70%) PABA = *p*-Aminobenzoic acid

*Correlation between the two parameters was high: $r = 0{\cdot}87$; $P > 0{\cdot}001$ but $< 0{\cdot}01$.

†Containing aluminium chlorhydroxide–zirconium chlorhydroxide–glycine complex.

Table 2. *Comparison of cumulative irritation scores in man and rabbit using 21-day occlusive test in man and 16-day uncovered test in the rabbit*

Compound or active ingredient	Vehicle	Concn (%)	Mean cumulative irritation score* Man	Rabbit
Group I—Antimicrobials and preservatives				
Zn pyrithione	P	0·05	0·0	—
		0·5	0·02	0·8
		2·0	0·1	9·5
Na pyrithione	P	0·05	0·0	2·8
		0·5	0·0	5·1
		2·0	2·9	37·8
MDS pyrithione	P	0·05	0·0	—
		0·5	0·0	6·6
		2·0	0·0	12·0
Hexachlorophene	P	0·03	0·0	0·6
		0·3	0·4	7·1
		3·0	10·4	44·1
Group II—Sunscreens				
PABA	P	1·0	0·0	4·1
		10·0	0·0	3·5
Homosalate	P	1·0	0·3	11·8
		10·0	1·3	10·5
Sulisobenzone	A	1·0	8·6	0·3
		10·0	53·1	3·6
Cinnamate (Giv Tan F)	SO	1·0	0·09	16·1
		10·0	0·02	16·3
Menthyl anthranilate	SO	1·0	0·2	14·5
		10·0	0·6	20·3
Marketed preparations				
Coppertone	—	—	0·0	13·5
Beach Party Sun Tan Lotion	—	—	7·0	23·3
Group III—Acids and alkalis				
Salicylic acid	P	0·1	0·1	1·0
		1·0	4·4	5·0
		10·0	6·7	4·8
Oxalic acid	P	0·1	0·4	1·6
		1·0	15·6	3·5
		5·0	67·1	1·6
Sodium hydroxide	W	0·05	0·0	0·0
		0·5	14·0	4·8
		1·0	37·0	34·0
		4·0	80·0	—
		5·0	80·0	64·0

P = Petrolatum A = Alcohol (70%) SO = Sesame oil W = Water

*Maximum scores: man, 84; rabbit, 64. The correlation between the scores in man and the rabbit was significant ($r = 0·87$; $P > 0·001$ but $< 0·01$) for Group I (Antimicrobials and preservatives).

Table 3. *Comparison of cumulative irritation scores in man and rabbit using 21-day occlusive test in man and 16-day uncovered test in the rabbit*

Compound or preparation	Vehicle	Concn (%)	Mean cumulative irritation score* Man	Rabbit
Group IV—Detergents				
Sodium lauryl sulphate	W	0·25	13·0	0·6
		1·0	17·0	8·0
		4·0	64·0	24·0
	P	1·0	30·0	6·5
Bubblebath (Mr. Bubble)	W	0·01	1·0	0·0
		0·1	0·03	0·0
		1·0	4·2	0·0
		10·0	45·2	2·5
	P	5·0	4·6	5·7

Table 3 (*Continued*)

Compound or preparation	Vehicle	Concn (%)	Mean cumulative irritation score* Man	Rabbit
Group V—Antiperspirants				
Aluminium chlorhydroxide complex	SO	3·0	0·0	7·3
	P	3·0	0·8	3·7
Marketed preparations				
Right Guard Super Dry†	—	—	0·6	47·3
Sure‡	—	—	2·3	31·3
Group VI—Vitamin E preparations				
Natural E-cream	—	12§	7·0	7·0
Natural Vitamin E oil	—	32§	0·0	12·0
Mennen E Deodorant	—	2·0§	3·9	31·0
Group VII—Cosmetics ingredients				
Ethanol	W	23	0·0	0·0
		70	0·0	0·0
		90	0·0	0·0
Mineral oil (new)	—	100	3·0	25·0
(old)	—	100	1·0	—
Petrolatum	—	100	—	1·0
Deo Base Kerosene	—	100	0·0	17·1
	MO	10	0·0	—
		50	0·0	—
Group VIII—Marketed skin-cosmetic preparations				
Ponds Cold Cream: Test no. 1.	—	—	1·0	23·5
Test no. 2.	—	—	0·06	24·8
Ultra Sheer Make Up Moisturizer	—	—	7·0	15·5
Vaseline Intensive Care	—	—	13·0	26·5
Noxzema	—	—	4·0	11·8
Group IX—Antipsoriatic drug				
Anthralin	P	0·01	9·5	25·5
		0·1	27·1	56·0

P = Petrolatum W = Water SO = Sesame oil MO = Mineral oil

*Maximum scores: man, 84; rabbit, 64.
†Containing aluminium chlorhydroxide complex.
‡Containing aluminium chlorhydroxide–zirconium chlorhydroxide–glycine complex.
§Vitamin E content (%).

abraded and intact skin, respectively, were 29 and 31 with Mennen E Deodorant, 3 and 1 with petrolatum and 24 and 25 with mineral oil (new).

The Draize test for skin irritation, more widely used than any other, consists of a single application of the test material on rabbits under occlusion. This test may be most useful in identifying substances that may irritate skin after accidental contact. A comparison of results obtained by this technique and by the present methods is shown in Table 4. These results, while exploratory, suggest that the multiple-application techniques may provide more definitive information about chronically applied substances that have a relatively mild irritation potential.

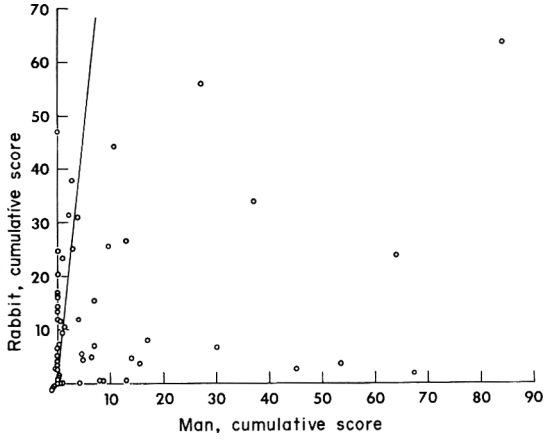

Fig. 3. Comparison of cumulative irritation scores from 21-day occlusive test in man and 16-day test (uncovered) in the rabbit.

DISCUSSION

In developing an animal test for wide use in evaluating the skin-irritation potential of ingredients and final formulations of drugs and cosmetics intended for prolonged use, the following considerations obtain: the test should be relatively simple to perform and to evaluate, findings should be reproducible and the results should have some degree of application to human use conditions. The present test has some deficiencies, but it appears to fulfil these requirements to a reasonable extent. In those tests where rabbit skin is more responsive to the test material than is human skin, the results may provide a useful margin of safety. As no test can serve as an absolute standard,

Table 4. *A comparison of results obtained on rabbits, using a single application (Draize, occluded) or 16 applications (cumulative irritation, uncovered) and on man, using 21 applications (cumulative irritation, occluded)*

Test material	Concn (%)	Draize primary irritation index* on rabbit	Mean cumulative irritation scores	
			Rabbit	Man
Sodium lauryl sulphate	4	0	24	64
	0·25	0	0·6	13
Ethanol	70	0	0	0
	92	0	0	0
Mineral oil	—	0·25	25	3
Petrolatum	—	0	1	—

*Tests performed by Ms Anne M. Wolven, Alza Research Corp., Palo Alto, Cal. (22 August 1974).

new ingredients and new formulations are best evaluated by comparison with known reference materials in parallel tests. Both intact and abraded skin should be studied, as in some cases the skin reaction may be very different when skin is deprived of its protective layer (stratum corneum).

Tabachnick & LaBadie (1973) reported a dramatic increase in epidermal deoxyribonuclease 24 hr after the hair of guinea-pigs had been close-clipped. This suggests that clipping may affect the results obtained with some topically applied materials. For this reason, in these rabbit studies, skin applications were made 48 hr after clipping. It may be advantageous to clip fast-growing hair more frequently to ensure proper scoring. The biochemical changes produced in the skin by clipping were considered in connexion with the already mentioned exploratory studies on hairless animals. We believe that extensive studies to ascertain the effect of clipping and shaving on skin physiology are in order (Baker, Maibach & Park, 1974).

Since these studies were conducted over a period of 11 months, season may inadvertently have played some role in the results. This factor has not been adequately evaluated.

Rostenberg (1957) has discussed genetics as a factor in allergenic and irritant skin reactions. As with any biological test, one must always be aware of the possible effects of genetic-related variations in response. Six animals were felt to be an adequate (but minimum) number to represent an animal test population, and 16 observations an adequate number for each animal (96 observations).

The data in Table 1 show that visual skin changes (capillary dilation) in rabbits correlate well with skinfold thickness (capillary leakage), indicating that either of these factors measures skin irritation. The correlation is not perfect; when the relation fails, it is probably due to the fact that visual skin changes may underestimate the actual extent of inflammation. This suggests that alteration in capillary permeability may be the more important component of the inflammatory response. Both parameters need to be monitored to ensure a proper evaluation of skin irritancy.

Exploratory studies were conducted to find out whether the skin-prostaglandin level could be used as an objective measure of skin irritation (Marzulli & Maibach, unpublished data 1974), but this did not prove successful. Grinding of the skin to a powder at the temperature of liquid nitrogen using a Spex grinder (Spex Industries, Metuchen, N.J.) was followed by assay of prostaglandin E with a radioimmunoassay kit (supplied by Clinical Assays, Inc., Cambridge, Mass.). This technique revealed no differences in prostaglandin E in irritated and control skin, about 1–2 ng/mg tissue-nitrogen being found in both control and irritated full-thickness rabbit skin.

We emphasize that the value of exaggerated irritancy assays (by the standard Draize technique or the two procedures described here) are a tool for comparison and not an absolute index. By referring this type of information to experience of human use, the investigator may be able to ascertain whether the irritancy potential of a test material is more or less than that of a use standard or is similar to it.

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APPENDIX*

At the conclusion of these experiments, representative animals were selected for histological examination.

*By Dr. R. B. Stebbins, Pathologist.

The test sites were scored (0–4 as described on p. 534) and biopsied, the tissue being mounted on cardboard and fixed in Bouin's solution. Paraffin-wax sections were stained with haematoxylin–eosin, examined by light microscopy and evaluated. The microscopic findings and their significance are summarized in Table 5.

These findings are considered to be exploratory, but they show certain trends. Test-site lesions scored as zero were accompanied by negative microscopic findings in 15 of 17 cases, while negative microscopic findings were accompanied by scores of zero in 15 of 27 cases. Significant microscopic changes were recorded for five skin specimens, including both sites tested with 3% hexachlorophene and two of four sites tested with Right Guard. Skin scores of 4 were recorded in each of these cases, but 4% sodium lauryl

Table 5. *Gross appearance and microscopic evaluation of rabbit skin at conclusion of 16-day skin-irritation test with substances listed in Experimental section*

Test substance	Skin score*	Microscopic evaluation†	No. of specimens‡	Comments§
Mineral oil	0	Negative	2	
Vitamin E, 2% (*dl*-α-tocopherol)	0	Negative	2	
Sodium lauryl sulphate, 1%	1	Epidermal thickening	1	NS
	1	Negative	1	
4%	0	Negative	1	
	2	Negative	2	
	2	Focal chronic slight epidermal inflammation	1	NS
	0	Focal epidermal necrosis	1	Sig
Sodium hydroxide, 1%	0	Negative	2	
	1	Negative	2	
	3	Negative	1	
	3	Focal acute and chronic slight epidermal inflammation	2	NS
Steroid cream	1	Negative	1	
	3	Negative	2	
Beach Party Sun Tan Lotion	2	Epidermal thickening; chronic cell infiltrate	1	Sig (?)
	3	Chronic cell infiltrate; marked epidermal proliferation	1	Sig (?)
Hexachlorophene, 3%	4	Focal dermal necrosis	2	Sig
Bubble bath, 5%	1	Chronic cell infiltrate	2	Sig (?)
Oxalic acid, 5%	0	Negative	2	
	1	Negative	1	
	2	Increased chronic cells under epidermis; nuclear pyknosis	1	NS
Sodium pyrithione, 0·5%	0	Negative	3	
	1	Increased chronic cells under epidermis; nuclear pyknosis	1	NS
Aluminium chlorhydroxide: in sesame oil, 3%	0	Negative	2	
	1	Negative	1	
	0	Increased chronic cells under epidermis; nuclear pyknosis	1	NS
in petrolatum, 3%	0	Negative	1	
	1	Epidermal thickening	1	Sig (?)
Right Guard with aluminium chlorhydroxide complex	4	Epidermal thickening	2	Sig (?)
	4	Epidermal thickening; chronic cell infiltrate; occasional eosinophils	2	Sig
Sure with aluminium chlorhydroxide and zirconium	3	Negative	1	
	2	Increased chronic cells	1	NS
	3	Epidermal thickening	2	NS

NS = Not significant Sig = Significant change Sig (?) = May be significant

*For definition of scores 0–4 see p. 534.

†Haematoxylin and eosin.

‡No. of skin specimens with the findings described.

§It is not known to what extent these skin changes may be reversible nor whether continued application would lead to necrosis of the integument.

sulphate produced focal epidermal necrosis in one of five animals and the lesion in this instance was scored as zero. The positive findings obtained with Right Guard concentrate may suggest a potential for skin irritation, although since the product was not tested as it is normally used (as a spray), one must not interpret this as a definitive evaluation of the marketed product.

It is possible that a more extensive use of histology of irritant dermatitis in prospective irritancy protocols might help to separate some of the variables involved.

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SHORT PAPERS

AN IMPROVED SEMI-QUANTITATIVE METHOD FOR THE ESTIMATION OF AFLATOXIN M_1 IN LIQUID MILK

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Introduction

All methods for the estimation of aflatoxin M_1 in milk involve extraction with organic solvents. In some methods, fresh milk or reconstituted powder is shaken with a suitable extractant so as to precipitate proteins and extract the toxin simultaneously, while in others a separate protein precipitant is used. In no method is protein precipitation complete, however, and at the extraction stage there is always a risk of emulsion formation. When this happens the analysis can become very prolonged and the recoveries poor.

The present modification to the method of Roberts & Allcroft (1968) eliminates this risk, reduces the working time for the analysis and increases its overall sensitivity.

Experimental

Protein precipitation and membrane clean-up. Liquid or reconstituted powdered milk (50 ml) is thoroughly mixed with acetone (100 ml) and then transferred to a dialysis sac prepared from water-washed Visking tubing (approximately 60 × 2 cm in diameter). The filled sac is carefully bent double and placed in a Kilner or similar jar containing 66% aqueous acetone (500 ml) and allowed to equilibrate overnight at room temperature. Mechanical shaking may be used but it does not seem to be essential.

Extraction. The dialysis sac is removed, allowed to drain into the jar and discarded. The diffusate is transferred to a 1-litre separating funnel and the toxin is extracted with chloroform (at least 250 ml). No emulsions occur and clean separations can be expected at this stage. The extract is passed through a bed of anhydrous Na_2SO_4 contained in a filter funnel lined with Whatman No. 41 paper. Two further extractions are made using 50 ml chloroform, the extract being passed each time through the bed of anhydrous Na_2SO_4. The combined chloroform extracts are evaporated to near dryness in a rotary evaporator, the last few millilitres being washed into a small glass vial with a small volume of chloroform. Evaporation is then completed in a stream of nitrogen. The residue (equivalent to 38·5 ml milk) is redissolved in 50–300 μl chloroform, depending upon the likely level of aflatoxin M_1 contamination.

Thin-layer chromatography. The semi-quantitative estimation of aflatoxin M_1 in the extract is carried out by visual matching under ultraviolet light (365 nm). Thin-layer plates coated with silica gel GHR (about 0·25 mm thick) are used and chromatograms are developed with chloroform–acetone–propan-2-ol (85:10:5, by vol.) in lined equilibrated tanks. This solvent system satisfactorily separates aflatoxin M_1 (R_F 0·48) from non-specific fluorescing spots. Standard solutions of pure aflatoxin M_1, obtained from Dr. P. L. Schuller (Bilthoven, Netherlands), was used during the development of this method.

Sensitivity. The smallest quantity of aflatoxin M_1 detectable under these conditions is about 0·5 ng and it may be calculated that, when the final volume of extract is 50 μl, the analytical limit of this method is in the region of 0·1 μg/litre milk.

Results and Discussion

Naturally contaminated whole milk in the form of roller-dried powder was reconstituted and diluted with uncontaminated fresh milk to give a series of milk samples with aflatoxin M_1 levels ranging from 0·1 to 9·0 μg/litre, as determined by the method of Roberts & Allcroft (1968). Twenty-three samples were analysed by the above procedure and the mean percentage estimate of expected values was 92·25% with a coefficient of variation of 13·65%. This may be compared with the results of a recent IUPAC-sponsored collaborative trial (Purchase, Stubblefield & Altenkirk, 1974) in which various liquid milk samples were analysed by the method of Jacobson, Harmeyer & Wiseman (1971) with coefficients of variation of 33·9–108·4% and with those of Schuller, Verhülsdonk & Paulsch (1973), whose own two-dimensional thin-layer chromatographic procedure had a coefficient of variation of 4–8% at an aflatoxin M_1 concentration of 0·1 μg/litre. The small losses of toxin in the present procedure can be ignored because the discrepancy is small compared with the known reproducibility of visual matching procedures used in this and the original (Roberts & Allcroft, 1968) semi-quantitative method.

The membrane clean-up step is taken from a procedure recently described for the multi-mycotoxin analysis of animal feedstuffs (Patterson & Roberts,

1975; Roberts & Patterson, 1975); its application to the analysis of milk for the aflatoxin metabolite M_1 confers three major advantages. Firstly, the risk of emulsion formation at the extraction stage is completely eliminated. Secondly, because of the ease of extraction, the actual working time for analysis prior to thin-layer chromatography is cut drastically. Thirdly, as a direct result of the new clean-up step, the residue is so small at the completion of the extraction that it may be redissolved in as little as 50 μl chloroform, thus increasing the sensitivity of the method by some 30 times compared with the original procedure (Roberts & Allcroft, 1968).

The detection limit of 0·1 μg/litre compares favourably with that of the more elaborate procedure of Schuller *et al.* (1973) and at least this level of sensitivity is currently required for any prospective milk surveys. Thus, recent proposals within the EEC for an upper permissible limit of 0·02 ppm for aflatoxin B_1 in complementary dairy feedstuffs would imply that, based on the daily feeding of 10 kg concentrates, an average 1% 'carry-over' of toxin into milk (cf. Kiermeier, 1973) and the daily production of 40 litres, no more than 0·05 μg aflatoxin M_1/litre could be expected in milk under normal circumstances.

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ABSENCE OF CARCINOGENIC ACTIVITY IN BD RATS AFTER ORAL ADMINISTRATION OF HIGH DOSES OF BISMUTH OXYCHLORIDE

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Summary—The pearlescent white pigment, bismuth oxychloride, which is used as a colouring agent for decorative cosmetics, was administered to BD rats in the diet in a concentration of 1, 2 or 5% for 2 yr. Neither carcinogenic activity nor other toxic effect attributable to the test compound was detected in the animals, which were maintained on a control diet from the termination of treatment until their death.

Introduction

The inorganic pigment, bismuth oxychloride (BiOCl; CI (1956) no. 77,163; Schultz no. 1415) is used extensively as a pearlescent white colouring (C-Weiss 10) in cosmetic products and particularly in decorative cosmetics, such as lipsticks, powder and nail enamel. Studies on the acute toxicity of the pigment (LD_{50} = 21·5 g/kg body weight, in rats), its subacute (90-day) toxicity and its skin and mucous-membrane tolerance revealed no toxic effects (unpublished data, cited from Mitteilung 3 of the Deutsche Forschungsgemeinschaft, Farbstoff-Kommission, 1968). The latter document classified BiOCl as a 'C' colouring for application on or near mucous membranes, and it was therefore necessary for a long-term feeding study to be carried out. We briefly report here the results of feeding a diet containing 1, 2 or 5% BiOCl to BD rats for 2 yr.

Experimental

The BiOCl used was a representative mixture of commercial preparations of the product. Its purity conformed to the criteria of the Deutsche Forschungsgemeinschaft, Farbstoff-Kommission (1968). The substance was a fine white powder, insoluble in water but soluble in mineral acid.

A homogeneous diet was freshly prepared every day in the form of a relatively solid mash containing Altromin® animal feed in powder form, sugar, Livio oil and BiOCl. Treatment was carried out on 7 days/wk, the diet being supplemented at weekends with salad and carrots.

Groups of 20 male and 20 female BD rats of the Institute's inbred strain (100 days old at the start of the test) were fed diet containing 1, 2 or 5% BiOCl. A group of 30 males and 30 females served as untreated controls. The daily intakes of the mash were 50 g for the males and 40 g for the females. After a feeding period of 2 yr the treatment was terminated and surviving animals were transferred to the basic Altromin diet and observed until their natural death.

Body weight was recorded monthly. At autopsy, all important organs (including the brain and nervous system) were examined, and tissues were fixed in 4% formalin for histological investigation.

Results and Discussion

The results of the study are summarized in Table 1. Oral administration, even of very high doses of

®Registered trade name of Altromin GmbH, Lippe, Germany.

Table 1. *Total doses administered, mean survival times and tumours found after the feeding of BiOCl to BD rats for 2 yr*

Dietary level (%)	No. of rats/group*	Total dose of BiOCl administered (g/kg body weight)		Mean survival time (days)	Tumours observed	
		Males	Females		Benign	Malignant
0	60	0	0	890^{+45}_{-30}	Mammary fibroadenoma (2) Hypophyseal adenoma (2)	Mammary carcinoma (1)
1	40	350	280	810^{+130}_{-90}	Mammary fibroadenoma (2)	
2	40	700	560	810^{+110}_{-80}	Mammary fibroadenoma (1)	
5	40	1750	1400	820^{+110}_{-80}	Mammary fibroadenoma (2) Hypophyseal adenoma (1)	

* Equal numbers of males and females in each group.

BiOCl, was well tolerated. The mean body weights of the test groups did not differ significantly from those of the controls. The total doses of BiOCl consumed were very high, amounting in the males on the highest dietary level to 1750 g/kg body weight (Table 1). The mean survival times for the groups of treated animals also corresponded approximately to those of the controls, but with much larger deviations around the mean value.

No macroscopic or histological findings could be attributed to the BiOCl treatment, and the types and incidence of tumours observed were closely comparable in the test and control groups. The mammary fibroadenomas and hypophyseal adenomas seen are spontaneous tumours characteristic of this strain. BiOCl thus seems to be non-carcinogenic in rats after oral administration even at high dose levels.

These results are in complete agreement with earlier investigations of the carcinogenic effect of other bismuth compounds. Osswald (1968) showed that feeding 2% bismuth subcarbonate ($[BiO]_2CO_3 \cdot 0{\cdot}5\ H_2O$) in the diet to BD rats did not induce tumours. Innes, Ulland, Valerio, Petrucelli, Fishbein, Hart, Pallotta, Bates, Falk, Gart, Klein, Mitchell & Peters (1969) found no increase of tumour incidence in two strains of mice fed the fungicide, bismuth dimethylthiocarbonate. Bismuth dextran did not produce local tumours on sc or im injection in the mouse or hamster (Haddow, Dukes & Mitchley 1961; Haddow & Horning, 1960).

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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EUGENOL

Synonyms: 4-Allyl-2-methoxyphenol; 4-allylguaiacol; 2-methoxy-4-prop-2-enylphenol; 1-hydroxy-2-methoxy-4-allylbenzene.

Structure: $HO \cdot C_6H_3(OCH_3) \cdot CH_2 \cdot CH{:}CH_2$.

Description and physical properties: *Givaudan Index* (1961).

Occurrence: Eugenol is the main constituent of several important essential oils such as oil of clove, clove stem and leaf, pimenta berry and leaf, bay and cinnamon leaf. Eugenol also occurs in smaller quantities in numerous other oils, including cinnamon bark, cananga, calamus and ylang ylang (Gildemeister & Hoffman, 1966; Guenther, 1949).

Preparation: By isolation from clove-bud oil, clove-leaf oil, cinnamon-leaf oil or Brazilian oil of *Ocimum gratissimum* (Bedoukian, 1967).

Uses: In public use since before the 1900s. Use in fragrances in the USA amounts to less than 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·005	0·01	0·40
Maximum	0·50	0·03	0·30	0·80

Analytical data: Gas chromatogram, RIFM no. 2962; infra-red curve, RIFM no. 2962.

Status

Eugenol was granted GRAS status by FEMA (1965) and is approved by the FDA as GRAS for food use (21 CFR 121.101). The Council of Europe (1974) included eugenol in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health, giving an ADI of 5 mg/kg. The *Food Chemicals Codex* (1972) and the *United States Pharmacopeia* (1965) both have monographs on eugenol. The Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for eugenol giving a conditional ADI of 0–5 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} has been reported as 2·68 g/kg in the rat, 3·00 g/kg in the mouse and 2·13 g/kg in the guinea-pig (Hagan, Jenner, Jones, Fitzhugh, Long, Brouwer & Webb, 1965; Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). Elsewhere, the acute oral LD_{50} in rats was reported as 1·93 g/kg (Sober, Hollander & Sober, 1950). Rats given four daily oral doses of about 900 mg/kg showed minor liver damage consisting of discoloration, mottling and blunting of lobe edges (Taylor, Jenner & Jones, 1964).

Dogs given single oral doses of 250 or 500 mg eugenol/kg showed signs of intoxication including vomiting and two out of four dogs on the higher level died; no effect was seen at 200 mg/kg (Lauber & Hollander, 1950). Intravenous injection of eugenol, diluted 1–20 times, decreased transiently the systemic arterial blood pressure and myocardial contractile force, impaired motor activity and increased salivary flow. Large doses caused pulmonary oedema in some dogs given eugenol iv and produced rigidity of the hind limbs when given intra-arterially (Sticht & Smith, 1971). The stomachs of rats and guinea-pigs given oral doses of 150 mg eugenol/animal showed histological damage consisting of desquamation of the epithelium and punctate haemorrhages in the pyloric and glandular regions (Hartiala, Pulkkinen & Ball, 1966). Degenerative and reparative changes in the gastric mucous barrier were followed histologically after repeated application of a 5% eugenol emulsion to the mucosa of Heidenhain's pouch in dogs (Hollander & Goldfischer, 1949).

In tests on acute toxicity to mucous membranes, eugenol applied bilaterally to the ventral surface of the tongue of dogs for 5 min caused erythema and occasionally ulcers with a moderate diffuse inflammatory infiltration (Lilly, Cutcher & Jendresen, 1972).

Short-term toxicity. No liver damage was observed in rats fed eugenol at 1% in the diet for about 4 months (Taylor *et al.* 1964). Feeding of eugenol at 0, 0·1 or 1% in the diet to groups of ten male and ten female rats for 19 wk exerted no effect on growth, haematology or organ weights and histology (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). No adverse effect was observed in a group of 15 male and 15 female rats fed eugenol at 79·3 mg/kg body weight/day for 12 wk (Joint FAO/WHO Expert Committee on Food Additives, 1967).

In a group of 20 male rats given an initial oral dose of 1·40 g eugenol/kg which was gradually increased to 4·00 g/kg, eight rats survived 34 days and 15 rats lived long enough to receive the maximum dose. Slight enlargement of the liver and adrenals was

*The latest of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology* 1975, **13**, 449.

observed and histological examination of the forestomach revealed moderately severe hyperplasia and hyperkeratosis of the stratified squamous epithelium with focal ulceration. A small degree of osteoporosis was also seen (Hagan *et al.* 1965 & 1967).

Irritation. Eugenol tested at 8% in petrolatum produced a mild irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1971). A patch test using undiluted eugenol for 24 hr produced no reactions in 20 subjects (Katz, 1946).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Greif, 1967; Kligman, 1971).

Metabolism. No absorption of eugenol occurred within 2 hr of application to the intact shaved skin of mice (Meyer & Meyer, 1959). Following ip injection of [^{14}C]eugenol into rats, radioactivity was distributed in various organs and the presence of $^{14}CO_2$ in the expired air indicated the demethylation of eugenol (Weinberg, Rabinowitz, Zanger & Gennaro, 1972). Over 70% of an oral dose of eugenol was excreted in the urine of rabbits (Schröder & Vollmer, 1932).

Eugenol, administered ip to rats, had little or no effect on microsomal enzyme induction, as evidenced by measurements of pentobarbitone and ethanol sleeping times (Seto & Keup, 1969) and of hexobarbitone sleeping time, urinary ascorbic acid excretion and hepatic amidopyrine demethylation (Grübner, Klinger & Ankermann, 1972). The hydroxylation of dimethylamidopyrine and hexobarbitone by mouse-liver microsomes was weakly inhibited by eugenol *in vivo* (Jaffe, Fujii, Sengupta, Guerin & Epstein, 1968). Hexobarbitone narcosis and zoxazolamine paralysis were slightly prolonged in mice treated with eugenol (Fujii, Jaffe, Bishop, Arnold, Mackintosh & Epstein, 1970).

The inhibition by eugenol of glucuronic acid conjugation in the stomach of rats and guinea-pigs (Hartiala *et al.* 1966) and of dogs (Raussi & Hartiala, 1963) may have some bearing on the reported mucinogenic activity of eugenol and its beneficial effect on gastric-ulcer formation.

Additional published data

Eugenol showed weak tumour-promoting activity following its application to mouse skin subjected to initiating treatment with 7,12-dimethylbenz[*a*]anthracene (Van Duuren, Sivak, Segal, Orris & Langseth, 1966). Eugenol failed to potentiate gastric-tumour production by 20-methylcholanthrene in mice (Hitchcock, 1952).

In a test on 21 patients suffering from various dermatoses, several essential oils and their constituents, including eugenol, were tested and produced positive patch-test reactions (Woeber & Krombach, 1969).

Skin irritation and phototoxicity tests were carried out on various soap perfume preparations, including a preparation containing eugenol (Fujii, Furukawa & Suzuki, 1972).

Eugenol-containing preparations were tested for allergic skin reactions in guinea-pigs and in patients undergoing dental treatment with these preparations (Koch, Magnusson & Nyquist, 1971).

Eugenol showed weak cytotoxic activity against HeLa cells (Stoichev, Zolotovich, Nachev & Silyanovska, 1967).

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HYDRATROPIC ALCOHOL

Synonyms: Hydratropyl alcohol; α-methyl phenylethyl alcohol; 2-phenylpropan-1-ol.
Structure: $C_6H_5 \cdot CH(CH_3) \cdot CH_2OH$.
Description and physical properties: A colourless liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By catalytic hydrogenation of the corresponding aldehyde.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·01	0·1
Maximum	0·15	0·015	0·05	0·6

Analytical data: Gas chromatogram, RIFM no. 74-136; infra-red curve, RIFM no. 74-136.

Status

Hydratropic alcohol was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included hydratropic alcohol at a level of 1 ppm in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 2·3 ± 0·407 g/kg (McGee, 1974). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (McGee, 1974).

Irritation. Hydratropic alcohol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (McGee, 1974). Tested at 6% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1974).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 6% in petrolatum and produced no sensitization reactions (Kligman, 1974).

Metabolism. Hydratropic alcohol is oxidized to hydratropic acid, which is excreted as a glucuronide, but some 10–20% of the alcohol is directly conjugated and excreted as hydratropyl glucuronide (Williams, 1959).

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HYDRATROPIC ALDEHYDE

Synonyms: Hydratropaldehyde; 2-phenylpropionaldehyde; α-methyl phenylacetaldehyde.
Structure: $C_6H_5 \cdot CH(CH_3) \cdot CHO$.
Description and physical properties: EOA Spec. no. 100.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By the alkaline condensation of acetophenone and ethyl chloroacetate followed by decomposition of the resulting glycidate (Bedoukian, 1967).
Uses: In public use since the 1930s. 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	0·001	0·005	0·08
Maximum	0·15	0·015	0·03	0·6

Analytical data: Gas chromatogram, RIFM no. 70–32; infra-red curve, RIFM no. 70–32.

Status

Hydratropic aldehyde was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included hydratropic aldehyde at a level of 1 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on hydratropic aldehyde.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·65 g/kg (2·71–4·91 g/kg) by Weir (1971) and as 2·8 g/kg by Jenner, Hagan, Taylor, Cook & Fitzhugh (1964). The acute dermal LD_{50} value in rabbits was reported as >5 g/kg (Weir, 1971).

Irritation. Hydratropic aldehyde applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was severely irritating (Weir, 1971). It was moderately irritating to rabbit skin in a primary skin irritation study (Weir, 1971). Tested at 2% in petrolatum, it produced a mild irritation after a 48-hr closed-patch test on human subjects (Kligman, 1971).

Conjunctival irritation produced by undiluted hydratropic aldehyde in the rabbit eye cleared by 24 hr (Weir, 1971).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Kligman, 1971).

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HYDROXYCITRONELLAL DIMETHYLACETAL

Synonym: 1,1-Dimethoxy-3,7-dimethyl-octan-7-ol.
Structure:
$CH_3 \cdot (OH)C(CH_3) \cdot [CH_2]_3 \cdot CH(CH_3) \cdot CH_2 \cdot CH(OCH_3) \cdot OCH_3$.
Description and physical properties: EOA Spec. no. 148.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From hydroxycitronellal and methyl alcohol in the presence of a catalyst (Bedoukian, 1967).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 4000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·03	0·5
Maximum	0·25	0·025	0·2	2·0

Analytical data: Gas chromatogram, RIFM no. 72–163; infra-red curve, RIFM no. 72–163.

Status

Hydroxycitronellal dimethylacetal was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed hydroxycitronellal dimethylacetal, giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on hydroxycitronellal dimethylacetal.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Shelanski, 1973).

Irritation. Hydroxycitronellal dimethylacetal applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Shelanski, 1973). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a 10% concentration in petrolatum and produced no sensitization reactions (Kligman, 1972).

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HYDROXYCITRONELLAL-METHYL ANTHRANILATE

Synonyms: Methyl-*N*-3,7-dimethyl-7-hydroxyoctylidene anthranilate; hydroxycitronellylidene-methyl anthranilate Schiff base.

Structure: $CH_3 \cdot (OH)C(CH_3) \cdot [CH_2]_3 \cdot CH(CH_3) \cdot CH_2 \cdot CH{:}N \cdot C_6H_4 \cdot OCO \cdot CH_3$.

Description and physical properties: *Givaudan Index* (1961).

Occurrence: Has apparently not been reported to occur in nature.

Preparation: By condensation using methyl anthranilate and hydroxycitronellal (Arctander, 1969).

Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·1
Maximum	0·1	0·01	0·03	0·6

Analytical data: Infra-red curve, RIFM no. 71–32.

Status

Hydroxycitronellal-methyl anthranilate is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), nor in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as >5 ml/kg (Lynch, 1971). The acute dermal LD_{50} value in rabbits was reported as >10 ml/kg (Lynch, 1971).

Irritation. Hydroxycitronellal-methyl anthranilate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Lynch, 1971). Tested at 6% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 6% in petrolatum and produced no sensitization reactions (Kligman, 1971).

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Lynch, T. A. (1971). Report to RIFM, 16 June.

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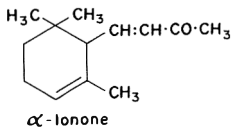
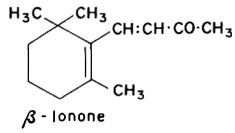
IONONE

Synonym: A mixture of α and β-isomers of cyclocitrylidenacetone.

Structure:



α-Ionone



β-Ionone

Description and physical properties: EOA Spec. no. 61.

Occurrence: The α-isomer has been reported in the essential oil of *Sphaeranthus inducus* L. and in the absolute essence of *Acacia farnesiana.* The β-isomer has been reported to be found in raspberry, in the distillate from flowers of *Boronia megatisma* Nees., and in a few other essences (*Fenaroli's Handbook of Flavor Ingredients*, 1971). α-Ionone occurs in the essential oils

of orange and *Ligusticum elatum*, in extract of *Osmanthus fragrans* Lour., in the flavour of tea, and in the essential oil of tangelo (*Citrus reticulata* Blanco × C. *paradisi* MacFayden). β-Ionone is an important constituent of essential oils of *Cunila lythrifolia* Benth., and *Siparuna nicaraguensis* Heml.; it has also been found in tomatoes (Naves, 1971).

Preparation: By chemical synthesis or by condensing citral with acetone to form pseudo-ionone which is then cyclized by acid-type reagents (Bedoukian, 1967).

Uses: In public use since the 1900s. Use in fragrances in the USA amounts to approximately 200,000 lb/yr.

Concentration in final product (%):

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

Status

Both α- and β-ionone were granted GRAS status by FEMA (1965) and are approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed α- and β-ionone, giving ADIs of 0·1 mg/kg for both. The *Food Chemicals Codex* (1972) has monographs on α- and β-ionone and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published monographs and specifications for both isomers, giving conditional ADIs of 0–0·1 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 4·59 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The ip LD_{50} value in mice was reported as 2·27 g/kg (Sporn, Schoebesch, Marin, Panaitescu & Runcanu, 1963).

Subacute toxicity. In feeding studies, 1000, 2500 and 10,000 ppm fed to rats in the diet for 17 wk produced microscopic liver changes, ranging from very slight swelling of the parenchymal cells at the lowest level to moderate swelling at the highest level (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). Rats fed 13–115 mg of α- and β-ionone for 5–9 days produced fatty infiltration of liver parenchymal cells (Shillinger, 1950). In a 12-wk feeding study in rats the concentration without effect was reported as 10·6 mg/kg for α-ionone and as 11·4 mg/kg for β-ionone (Bär & Griepentrog, 1967).

Irritation. A patch test using ionone full strength for 24 hr produced no reactions in eleven subjects (Katz, 1946). Mendelsohn (1946) reported ionone to be non-irritating.

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Greif, 1967).

Metabolism. Ionones are metabolized mainly by oxidation of the ring system at the carbon atom alpha to the ring double bond and by reduction of the carbonyl group (Williams, 1959). On administration to dogs α-ionone is hydroxylated in the ring at the carbon atom which is alpha to the ring double bond to yield 5-hydroxy-α-ionone (Prelog, Wursch & Meier, 1951). Rabbits dosed orally with β-ionone excreted in the urine unchanged β-ionone, 3-oxo-β-ionone, 3-oxo-β-ionol, dihydro-3-oxo-β-ionol and 3-hydroxy-β-ionol. Excretion products were isolated as 2,4-dinitrophenylhydrazone derivatives and as *p*-nitrobenzoate derivatives. The glucuronides of 3-oxo-β-ionol and dihydro-3-oxo-β-ionol were also detected in the urine (Ide & Toki, 1970).

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α-IRONE

Synonyms: 6-Methyl-α-ionone; *cis*-(2,6)-*cis*-(2^1,2^2)-α-irone.
Structure: $HC_3 \cdot C_9H_{15} \cdot CH{:}CH \cdot CO \cdot CH_3$.
(For the structure of the α isomer of ionone, $C_9H_{15} \cdot CH{:}CH \cdot CO \cdot CH_3$, see monograph thereon, p. 549.)
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Found in orris root oil, where the β- and γ-isomers also occur as well as several stereo-isomers (*Givaudan Index*, 1961). It is also found in raspberry and some flowers of Pittosporum sp. (*Fernaroli's Handbook of Flavor Ingredients*, 1971).
Preparation: Synthetically from dimethylheptenone.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·0005	0·002	0·04
Maximum	0·03	0·003	0·01	0·5

Analytical data: Gas chromatogram, RIFM no. 72–26; infra-red curve, RIFM no. 72–26.

Status

α-Irone was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed α-irone, giving an ADI of 0·1 mg/kg.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Shelanski, 1972).

Subacute toxicity. In a 12-wk feeding study on rats, the concentration without effect was reported as 5·2 mg/kg (Bär & Griepentrog, 1967).

Irritation. α-Irone applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Shelanski, 1972). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1972).

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ISOAMYL ACETATE

Synonyms: Amyl acetate; β-methyl butyl acetate.
Structure: $CH_3 \cdot CH(CH_3) \cdot [CH_2]_2 \cdot OCO \cdot CH_3$.
Description and physical properties: EOA Spec. no. 110.
Occurrence: Reported to be found in a number of naturally occurring products, including apple, banana, cocoa bean, coffee, cognac, grape, peach, pear, pineapple and strawberry (FEMA, 1974).
Preparation: By the esterification of commercial isoamyl alcohol with acetic acid.
Uses: In public use before the 1900s. Use in fragrances in the USA amounts to about 10,000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·003	0·05
Maximum	0·2	0·02	0·02	0·3

Analytical data: Gas chromatogram, RIFM no. 72–164; infra-red curve, RIFM no. 72–164.

Status

Isoamyl acetate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) listed isoamyl acetate, giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on isoamyl acetate and Browning (1965) has an extensive monograph on amyl acetate.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973). The oral (by stomach tube) LD_{50} value in rabbits was reported as 57 mmol/kg (Munch, 1972).

Irritation. Isoamyl acetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1973). Tested at 8% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

Threshold limit value. The threshold limit value for isoamyl acetate has been set at 100 ppm (American Conference of Governmental Industrial Hygienists, 1973).

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Munch, J. C. (1972). Aliphatic alcohols and alkyl esters. Narcotic and lethal potencies to tadpoles and to rabbits. *Ind. Med. Surg.* **41**, 31.

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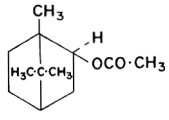
ISOBORNYL ACETATE

Synonyms: 2-Camphanyl acetate; bicyclo[2,2,1]-1,7,7-trimethylheptanyl-2-acetate.

Structure:



Description and physical properties: EOA Spec. no. 74.

Occurrence: Has apparently not been reported to occur in nature.

Preparation: By reaction of camphene with acetic acid under acid catalysis or by any other suitable means.

Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 200,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·005	0·2
Maximum	0·4	0·04	0·05	1·0

Analytical data: Gas chromatogram, RIFM no. 70–16; infra-red curve, RIFM no. 70–16.

Status

Isobornyl acetate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included isobornyl acetate in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on isobornyl acetate.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as > 10 g/kg (Fogleman, 1970). The acute dermal LD_{50} value in rabbits exceeded 20 g/kg (Fogleman, 1970).

Short-term toxicity. Isobornyl acetate dissolved in corn oil was administered daily to rats by stomach tube in doses of 0 (control), 15, 90 or 270 mg/kg body weight/day for 13 wk. There were no differences between treated and control animals in the rate of body-weight gain, the food intake or the results of haematological investigations. Male rats given 270 mg/kg/day showed a decrease in renal concentrating ability, an increase in water intake, exfoliation of renal tubular cells, increased kidney weight and vacuolation of the renal tubular cells. Signs of nephrotoxicity were also seen with daily doses of 90 mg/kg. Vacuolation of the epithelium of the intrahepatic bile-duct and an increase in liver weights were found at 270 mg/kg. The caeca were also enlarged at this dosage level. The no-effect level found was 15 mg/kg/day, more than 100 times the calculated maximum intake by man (Gaunt, Agrelo, Colley, Lansdown & Grasso, 1971).

Irritation. Isobornyl acetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Fogleman, 1970).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1970).

References

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Kligman, A. M. (1970). Report to RIFM, 7 October.

ISOBORNYL PROPIONATE

Structure: $C_{10}H_{17} \cdot OCO \cdot CH_2 \cdot CH_3$ (for structure of isobornyl radical, $C_{10}H_{17}$, see monograph on isobornyl acetate, p. 552).
Description and physical properties: A colourless oily liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By the reaction of camphene with propionic acid using an acid catalyst.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·005	0·2
Maximum	0·4	0·04	0·05	1·0

Analytical data: Gas chromatogram, RIFM no. 72–166; infra-red curve, RIFM no. 72–166.

Status

Isobornyl propionate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included isobornyl propionate, at a level of 2 ppm, in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. Isobornyl propionate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Moreno, 1973). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List 1, no. 412, p. 209. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavouring ingredient usage levels. No. 2163. *Fd Technol., Champaign* **19** (2), part 2, 155.
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Moreno, O. M. (1973). Report to RIFM, 1 February.

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ISOBUTYL BENZOATE

Structure: $C_6H_5 \cdot COO \cdot CH_2 \cdot CH(CH_3) \cdot CH_3$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By direct esterification of isobutyl alcohol with benzoic acid, under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·3
Maximum	0·3	0·03	0·05	1·0

Analytical data: Gas chromatogram, RIFM no. 72–167; infra-red curve, RIFM no. 72–167.

Status

Isobutyl 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 (1974) listed isobutyl benzoate, giving an ADI of 5 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·7 mg/kg (3·19–4·29 mg/kg) (Levenstein, 1973). The acute dermal LD_{50} value in rabbits was reported as > 5 ml/kg (Levenstein, 1973).

Irritation. Isobutyl benzoate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Levenstein, 1973). Tested at 2% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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Fd Cosmet. Toxicol. Vol. 13, pp. 555–563. Pergamon Press 1975. Printed in Great Britain

Review Section

THE INFLUENCE OF MILK AND RELATED DIETARY CONSTITUENTS ON LEAD METABOLISM

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Summary—For over a century milk was recommended unreservedly to counteract lead poisoning in industry. The value of this empirical practice was subsequently supported by a number of studies using whole milk and its separate principal constituents but, during the last two decades, industrial hygienists have endeavoured to abandon this preventive measure. The conflicting reports concerning the efficacy of milk as a prophylactic agent against lead probably stem from inattention to the composition of the diet of the animals under test, the modes of administration of lead and the total amount of lead given. However, on balance it appears that whole milk can no longer be regarded as a prophylactic agent.

Introduction

Observations made in the white-lead industry over a century ago indicated that milk caused the symptoms of lead poisoning to abate and, if taken regularly, prevented the occurrence of the disease in lead workers. A substantial number of early studies using whole milk and its separate principal components appeared to validate this prophylactic effect, but recent work seems to vitiate these earlier results and emphasizes the undeniable role of good industrial hygiene as the prime factor in the protection of lead workers. Indeed, it is highly likely that with a diet adequate in calcium, phosphorus and iron, gastro-intestinal absorption of lead is actually enhanced by whole milk.

In view of the exposure of large numbers of children to many sources of lead in the environment and the 'protective' effect of calcium and phosphate, it is important to recognize that whole milk may contain agents that promote the absorption of lead from the gut.

Studies using whole milk or milk products

The administration of milk to lead workers has a long history. It was strongly advocated by early writers (Feissinger, 1900; McKenna, 1913; Tanquerel des Planches, 1848) and was still recommended by Hunter in 1969 and by Krook in 1974. The basis for the advice in the early literature, however, is not readily apparent, although it may have been related to empirical observations that workers who were poorly fed developed lead poisoning more often than those who were relatively well fed, the milk being given to correct deficiencies in the diet*. Animal experiments, however, have demonstrated that milk can be of benefit in other ways. Thus, Miyasaki (1930) reported that milk considerably reduced the absorption of lead from the gut of mice, a finding later confirmed not only in the mouse (Tompsett, 1939) but also in the dog (Horwitt & Cowgill, 1939) and rat (Ardelean, Gontzea, Sutzescu, Vintila & Vaida, 1955). Newborn rats, which have a high level of intestinal lead absorption (Kostial, Šimonović & Pišonić, 1971a) showed a reduction in their body burden of lead when calcium chloride and potassium hydrogen phosphate were added to their diet of cows' milk until the level of calcium and phosphate was that characteristic of rats' milk (Kostial, Šimonović & Pišonić, 1971b). An early study by Weyrauch & Necke (1933) using rabbits was not decisive but Buckup, Böhm, Zimmermann, Remy, Portheiene & Voss (1956) concluded from their experiments that not only was less lead deposited in the skeleton of rabbits given milk than in those not given milk, but that haemoglobin levels were also maintained at higher levels.

A defect in a number of early studies was the use of small numbers of animals, as can be demonstrated by a consideration of the work of Aub, Fairhall, Minot & Reznikoff (1926). These authors concluded that milk had no influence on the absorption of lead from the gut in cats. An inspection of their data (Table 1), however, shows such a markedly idiosyncratic response that this conclusion seems hardly justified.

Strong evidence that milk has no protective action against lead was provided by Wittgens & Niederstadt

* It is of interest to note that Wetherill, Rabinowitz & Kopple (1974) have found that fasting increases gastrointestinal absorption of lead by a factor of eight.

Table 1. *Effect of milk on the rate of gastro-intestinal absorption of lead by cats*

Diet	Duration of experiment (days)	Total lead administered (g)	Total lead found in tissues (mg)	Lead (μg) retained/day (A)	Lead (mg) ingested/day (B)	B/A
With milk	43	3·78	16·00	372	87	234
	55	3·74	46·48	845	68	80
	95	4·72	23·00	242	49	202
	99	11·40	86·33	872	115	131
Without milk	18	2·58	24·85	1380	143	103
	30	2·70	38·07	1269	90	70
	66	7·14	28·89	437	108	247
	124	6·28	88·99	717	50	69

From Aub *et al.* (1926).

(1955). In their experiments, rats were given relatively large doses of lead acetate, either orally or by ip injection for a period of up to 8 months. A very high oral dose level of 2·3 g/kg/week had to be maintained for 15 weeks before any clearly defined evidence of lead poisoning was produced (cf. Ardelean *et al.* (1955) who used 0·04 g/kg/week). Rats given lead orally were denied fluid for a day and then each animal was given an exact measure of aqueous lead acetate and only after they had drunk all this solution were they allowed either water or milk. In this respect the study differs from the great majority of the animal experiments discussed in this review, in which the lead was presented mixed in the diet. The consequences of the surges in lead to which the rats were subjected is difficult to assess, but it might overwhelm a prophylactic agent, at least for part of the residence time in the gastro-intestinal tract. Furthermore, the milk-fed rats received, *ad lib.*, whole milk enriched with condensed milk, but no water. This regime would be likely to decrease the speed with which the material passed through the gut and hence increase the opportunity for lead absorption (Tompsett, 1939).

The criteria used by Wittgens & Niederstadt (1955) to diagnose signs of chronic lead poisoning in their animals included loss of weight, a decrease in haemoglobin, poor condition of fur and the appearance of degenerative changes in the liver and kidneys. Their contention was that these signs were more apparent in the animals fed milk than in those having only water. Their claim that there was no essential difference in the haematological findings between the different groups of animals may be a reflection of the authors' preoccupation with the gross effects associated with plumbism. In fact, in the group of animals given lead by the oral route, the haemoglobin level fell less in those that had milk than in those that did not (Table 2). An increased content of lead in the skeleton was observed in some rats, but was not regarded as being of diagnostic significance. In two of the three sub-groups of rats examined, the bone lead concentration was greatest in the animals given water, but none of the differences was significant because of the small number of analyses made.

Table 2. *Decrease in haemoglobin levels found in the rat study of Wittgens & Niederstadt (1955)*

Duration of treatment (wk)	Percentage decrease in haemoglobin after lead administration			
	Ip. with ingestion of		Orally, together with	
	Milk	Water	Milk	Water
6	30·00	21·11	—	—
12	26·25	22·14	—	—
15	—	—	4·37	7·22

On the basis of their findings, Wittgens & Niederstadt (1955) concluded not only that milk had no prophylactic effect against lead poisoning but that it actually enhanced lead toxicity and should not be given to lead workers. When considering their work, however, it is important to realize that the absorption of lead following ip administration is not affected by the calcium content of the gut (Lederer & Bing, 1940) and so there is no basis for supposing that milk will have any prophylactic effect when lead is given by this route. By the same token, milk is unlikely to affect the absorption of lead from the lung, which is the predominant route of absorption following occupational exposure. In the latter context, however, it is worth noting that part of the lead which is retained in the lungs can subsequently be conveyed to the gut by muco-ciliary transport and swallowing.

The qualitative observations of Wittgens & Niederstadt (1955) have been elegantly quantified by Kello & Kostial (1973) using radioactive lead in milk fed to rats. These authors demonstrated that ^{203}Pb in milk is more efficiently absorbed from the gastro-intestinal tract than that ingested in a milk-free diet and that the addition of milk to the diet has no influence on the absorption of ^{203}Pb injected into the peritoneal cavity. Unfortunately, in two of the experimental groups, the dietary intake of iron was low*, and this may have introduced some bias into the study. Furthermore, the study did not include a group of animals receiving a diet deficient in calcium and phosphorus.

The studies that report on the efficacy of milk as a prophylactic agent in human subjects have been confined to lead workers and they present conflicting points of view. Thus, Collier (1952), Dizon, Luciano, Navarro, Anselmo & Pesigan (1950), Schweigart

*Although Six & Goyer (1970) have demonstrated that iron-deficiency results in greater soft-tissue concentrations of lead, it is not clear whether this is a consequence of enhanced gastro-intestinal absorption.

(1957), Travers, Rendle-Short & Harvey (1956) and Troisi (1950) consider that milk does have some value as a prophylactic against lead poisoning whereas Boyadzhiev (1960), Lockhart (1963), Schiemann (1960) and Vigliani (1954) consider that it does not. A similar lack of agreement is to be found in four articles which review the whole question of the issue of milk to lead workers. Of the four, only that by Zielhuis (1960) recommends supplementing the diet with milk. Wittgens & Niederstadt (1954) consider the practice to be actually harmful, while Sand (1965) uses statistics from the second world war in his condemnation. The latter author suggests that the fall in the incidence of lead poisoning during the war could be correlated with a decrease in the amount of milk available in the diet. In view of the pressures on adequate medical supervision during this period, however, it seems more reasonable to suggest that the fall in the number of cases of lead poisoning was due to inadequate diagnosis; a similar trend was also observed during the 1914–1918 war, presumably for the same reason. The fourth review author, Longley (1967) considers that the provision of extra milk discourages good industrial hygiene; he is against its provision on these grounds.

Influence of separate constituents of milk on ingested lead

The identification of the milk components that promote the gastro-intestinal absorption of lead was sought by a consideration of some of its separate principal constituents (Table 3).

Calcium

Calcium has a marked influence on the effective toxicity of lead to dogs (Calvery, Laug & Morris, 1938), rats (Barltrop & Khoo, 1975; Grant, Calvery, Laug & Morris, 1938; Pletscher, Richterich, Thoelen, Lüdin & Staub, 1952), mice (Tompsett & Chalmers, 1939), pigs (Hsu, Krook, Shively, Duncan & Pond, 1973) and fish (Jones, 1938; Schroeder, 1965).

The influence of calcium on lead absorption was considered by Schroeder (1965) to be one example of a general rule that the absorption of trace metals by plants, lower animals, fish and mammals is inversely related to the concentration of calcium in the medium from which it is being absorbed. He put forward the hypothesis that there may be a cellular transport mechanism common to all living things which is saturated by calcium ions and which regulates the exchange of other cations from the immediate environment. There is some support for this hypothesis in the work of Tidball (1964), who has shown that magnesium and calcium loosely bound in the structure of the mucosal membrane regulate the aqueous permeability of the intestinal epithelium of the rat.

Table 3. *Constituents of milk considered to play a role in lead absorption and/or its metabolism*

Constituents	Average level (g/100 ml whole milk)
Calcium	0·125
Phosphorus	0·096
Casein	2·8
Other proteins	1·2
Fat	3·7
Vitamin C	0·0016
Citric acid	0·17
Lactose	4·8

From Macy & Kelly (1961).

Lederer & Bing (1940) highlighted one important variable in the consideration of milk prophylaxis when they showed, by ip injection of lead into rats, that the effect of calcium on the assimilation of lead is dependent mainly upon events occurring in the digestive tract. With peritoneal absorption, the retention of lead was independent of the calcium and phosphorus levels of the diet. These authors also showed that orally ingested lead was retained in much greater amount in bone and kidney (but not in liver) when the calcium content of the diet was high than when it was low.

The first clear reference to elevated blood-lead levels and marginal dietary adequacy among urban subjects was made by Six & Goyer (1970), who showed that in rats lead ingestion at a low level, not overtly harmful when accompanied by normal levels of calcium in the diet, resulted in toxic manifestations when the level of calcium was reduced.

It is pertinent to note that the lowest calcium content of the dog's diet shown by Calvery *et al.* (1938) to result in enhanced lead toxicity was similar to that of a large proportion of the modern American diet. This suggests that those persons with a diet low in calcium will have a greater risk of absorbing excessive amounts of lead from the gut than those in whom dietary calcium is normal (Lin-Fu, 1973). It is disturbing to note that there is evidence that, in the UK, dietary calcium is far from being at a universally satisfactory level (Brennan, 1972; Ministry of Agriculture, Fisheries and Food, 1971). The significance of this dietary inadequacy is greatly enhanced by the possibility that calcium deficiency may be involved in the production of lead pica. Snowdon & Sanderson (1974) found that weanling rats on a calcium-deficient diet voluntarily ingested toxic amounts of lead, and it would clearly be of considerable importance to establish whether this is also the case in children.

The influence of phytic acid on intestinal calcium absorption is also relevant to these considerations because of the very low solubility of its calcium salts (Holmes, Enoch, Taylor & Jones, 1973; Reinhold, Nasr, Lahimgarzadeh & Hedayati, 1973; Watney, Chance, Scott & Thompson, 1971; Wills, Day, Phillips & Bateman, 1972). A high phytate intake will minimize calcium absorption and hence, by a secondary effect, may promote the uptake of lead, although the possibility that lead has an equal affinity for phytate cannot be ruled out.

Calcium, phosphorus and vitamin D

When the effects of phosphate and vitamin D are minimized, the inter-relationship between calcium and lead is relatively well defined. If these variables are not satisfactorily controlled, however, a more complex situation can arise. Shelling (1932), for example, has produced evidence that diets high in

calcium render lead more toxic to rats than do diets high in phosphate. The rationale given for this effect closely parallels that given for the production of rickets in rats (McCollum, Simmonds, Parsons, Shipley & Park, 1921) by the use of a diet with a Ca/P ratio of about 4:1. The introduction of large amounts of calcium without phosphate into rats merely diverts the available phosphate to rid the body of the excess calcium as the phosphate and thus interferes with the formation of the relatively inert trilead phosphate (cf. Bischoff & Blatherwick, 1927). Phosphate also lowers plasma lead levels by promoting increased binding of peptized lead phosphate to erythrocytes (Clarkson & Kench, 1958) so rendering it inert; this is an important aspect of the lead integration process proposed by McRoberts (1973). Thus, there are two main areas of reaction, the soft tissues, especially blood, and the lumen of the gastro-intestinal tract. The relationship between these areas becomes apparent when the Ca/P ratio is raised, because although competitive inhibition by the elevated calcium level discourages absorption of lead, the lead that is absorbed is more likely to remain in the plasma. In applying his results to the prophylaxis of lead poisoning in man, Shelling (1932) drew attention to the treatment advocated by Aub, Fairhall, Minot & Reznikoff (1925) involving the use of milk containing a high concentration of available calcium and phosphate with a Ca/P ratio of about 1·3.

A more detailed study of the interrelationship between calcium, lead and phosphate was made by Sobel, Yuska, Peters & Kramer (1940) who showed that the addition of either calcium or phosphorus to rats fed a low-calcium, low-phosphorus diet containing lead will depress lead retention through a decrease in absorption from the gut (cf. Barltrop & Khoo, 1975). A theory was developed that the deposition of lead and calcium is independent and an adequate level of calcium and phosphorus at a Ca/P ratio between 1 and 2 renders lead deposition as ineffective as possible. These authors also showed that the addition of Vitamin D to any of the types of diet studied, which however excluded one with an ideal calcium–phosphorus intake, caused a rise in blood lead and enhanced lead retention. This work provided confirmation of an earlier study (Sobel, Gawron & Kramer, 1938), in which it was found that the presence of vitamin D in the diet of the rat approximately doubled lead absorption from the gut.

In animals with established lead poisoning, vitamin D behaves differently after lead administration has ceased, producing a fall in blood-lead concentration and a decreased loss of lead from bone. Under the same condition, a high-phosphorus, low-calcium diet produces the same effect whereas a high-calcium, low-phosphorus diet produces the opposite effect (Sobel & Burger, 1955).

It is noteworthy that Rapoport & Rubin (1941) have reported a direct association between the incidence of lead poisoning in children and the level of sunlight and supplementary vitamin D. Clearly, it is desirable to avoid undue supplementation with vitamin D when lead is being ingested, especially since it is possible that vitamin D may have an effective minimum intake at the most favourable relative and absolute levels of calcium and phosphorus.

Protein

The importance of casein in increasing the resistance of white rats to lead intoxication has been shown by Baernstein & Grand (1942) and by MacDonald, Ezmirlian, Spain & Rounds (1953). Similarly, Gontzea, Sutzesco, Cocora & Lungu (1964) showed that dietary protein combated the effects of sc injected basic lead acetate in rats. Rats fed a protein-deficient diet retained 40–73% more lead in the liver, spleen and tibia than rats receiving adequate protein. Kidney damage was also greater with the regime poor in proteins. Boyadzhiev (1959 & 1963) has also shown the importance of protein by demonstrating that rats on a low-protein, high-fat diet had more pronounced symptoms of lead poisoning than those on a high-protein diet. It is also significant that Buckup *et al.* (1956) found that rabbits receiving methionine and cysteine showed an increased resistance to lead and Niemöller (1957) used methionine as an inhalant prophylactic with beneficial effects. The use of serum albumin for the prevention of lead poisoning has been reported by Biondi (1959), De Renzi & Ricciardi-Pollini (1952), Merli (1957) and Odescalchi (1956), and the use of radio-lead by Matsukubo (1959) has demonstrated that it is bound by the albumin and globulin components of serum proteins in rats injected with lead salts containing ^{210}Pb.

Since a high-protein diet markedly increases the absorption of calcium (Harper, 1971), presumably through the formation of soluble complexes with amino acids, it might be thought that the same process could increase the absorption of lead, an effect which would be at odds with the apparent ability of protein to protect against the toxic effects of lead. However, the defence mechanism associated with protein probably involves sulphydryl groups, which facilitate excretion of lead like standard chelating agents. Indeed, the overall dynamics of such a system might provide a rationale for the observations of Milev, Satler & Menden (1970) and Barltrop & Khoo (1975) that both high and low dietary protein levels increased intestinal lead absorption in rats.

Fat

Tanquerel des Planches (1848) advocated the use of fat food in lead prophylaxis, but more recent authors (Boyadzhiev, 1960; Buckup *et al.* 1956; Zielhuis, 1960) have favoured a diet low in fat. Sand (1965) prescribed skimmed milk instead of whole milk for lead workers since fat was thought to solubilize ingested lead (Sand, 1965; Weyrauch & Necke, 1933), although Tompsett (1939) claimed that fat in the diet of mice did not appear to influence the absorption of lead from the gut.

The mechanism whereby fat solubilizes ingested lead has not been considered but it may involve the formation of the lead analogues of the readily absorbed bile–fatty acid–calcium complexes described by French (1942). However, Steggarda & Mitchell (1951) found that dietary fat had, within a wide range, no effect upon calcium retention in man.

The first guide to the degree of enhancement of lead absorption produced by fat was given by Weyrauch & Necke (1933), who found that oil and margarine increased the amount of lead absorbed by rabbits

by a factor of more than ten. However, they did not give the level of dietary calcium and this is clearly vital to a proper assessment of their results since mass law considerations are involved. Support for their observation has been provided by Barltrop & Khoo (1975) using ^{203}Pb in rats, although with the same technique, Kello & Kostial (1973) did not find a clear correlation between fat and gastro-intestinal absorption.

The relative water solubilities of the lead and calcium salts of stearic, oleic, palmitic and butyric acids do not afford clear support for the observation of Weyrauch & Necke (1933), but the lipid solubilities may give a better guide.

Vitamin C

The effects of vitamin C on lead toxicity are unclear. A number of workers (Buckup *et al.* 1956; Davydova, 1953; Gerlich & Remy, 1956; Gontzea, Dumitrache, Rujinski and Cocora, 1963; Hanusova & Michalova, 1956; Holmes, Campbell & Amberg, 1944; Matusevich, 1953; Mokranjac, Radmić and Soldatović, 1962; Odescalchi & Andreuzzi, 1959; Pillemer, Seiffer, Kuehn & Ecker, 1940; Remy, 1956; Wu, Lu & Chang, 1959) have indicated that vitamin C protects tissues against lead, while several others (Boyadzhiev & Tyutyulkov, 1960; Dannenberg, Widerman & Friedman, 1940; Evans, Norwood, Kehoe & Machle, 1943; Gontzea, Sutzescu, Stanciu & Lungu, 1964) advocate the opposite view. The problem, however, is somewhat academic, since, although the vitamin C content of 0·5 litre of fresh cows' milk supplies about 70% of the daily requirement for a child, very little (only about 1·8 mg/100 ml) remains after pasteurization.

Citric acid

The citric acid in milk may play a very minor supporting role in prophylaxis, in as much as it helps to create a high concentration of available calcium by virtue of its chelating power.

A complicating feature in the assessment of citrate as a minor aid against lead retention arises from the work of Kety (1942), who found that citrate chelates lead more efficiently than calcium. However, he suggested that citrate normally present in the blood will increase the mobilization and excretion of lead without increasing the concentration of the toxic lead ion.

Using large doses of sodium citrate, a number of authors (Hardy, Bishop & Maloof, 1951; Hsü & Yao, 1958; Kety & Letonoff, 1941; Moeschlin & Schechterman, 1952; Mokranjac, Radmić & Soldatović, 1958; Rossi, Vitacca & Pagano, 1954; Shibata, 1957; Shiels, Thomas & Palmer, 1950; Suntych, 1953) were able to combat lead poisoning in man and a variety of animals. In every study except that of Hardy *et al.* (1951) a marked increase in urinary lead excretion occurred. By contrast, Masuda (1959) found that potassium sodium citrate had only a minimal therapeutic effect on orally poisoned rabbits.

The finding of Niemöller (1957), that inhalation of aerosols of calcium sodium citrate and zirconium citrate had no prophylactic value for persons exposed to lead, supports the work of Schubert & White (1952). The latter authors showed that zirconium citrate caused at least a threefold increase in the urinary excretion of ^{210}Pb but only when injected simultaneously with the soluble lead salts. Similarly, Sano (1953) found that sodium citrate administered orally to guinea-pigs did not affect the urinary excretion of injected or inhaled lead. These observations can be rationalized by the fact that citrate is rapidly metabolized in the body via the Krebs cycle. The situation was elegantly demonstrated by Schubert & Lindenbaum (1960), who injected small amounts of monofluoroacetic acid into lead-poisoned rats so that citric acid accumulated as a result of the inhibition of the enzymes in the Krebs cycle. Injection of modest doses of sodium citrate then provided the animals with protection against lead poisoning.

Lactose

The enhancement of calcium absorption by dietary lactose has been known for many years and has been demonstrated in rats (Bergeim, 1926), dairy calves (Robinson, Huffman & Mason, 1929), dogs (Greenwald & Gross, 1929), chicks (Kline, Keenan, Elvehjem & Hart, 1932) and children (Mills, Breiter, Kempster, McCay, Pickens & Outhouse, 1940). Lengemann, Comar & Wasserman (1957) reviewed the subject and emphasized the favourable effect of milk on calcium absorption.

The enhancement of calcium absorption appears to be specific for lactose and xylose (Pansu & Chapuy, 1970), and a variety of mechanisms for this action have been advanced, ranging from purely physicochemical concepts (Herrington, 1934) to metabolic effects (Duncan, 1955; Mills *et al.* 1940). If a complex of ionic calcium and lactose were the sole agency of increased intestinal absorption, the relative stability constants of this complex and the lead analogue would need to be known in order to assess the total benefit of lactose in the presence of ingested lead.

Of the agents considered*, lactose, fat and citric acid appear to be capable of enhancing the absorption of lead, although the contribution of citrate (0·17 g/100 ml in milk) is likely to be very small and there is some doubt about the role of fat. It would be of great interest to study the activity of lactose, the most abundant of these agents in milk (4·8 g/100 ml), in promoting the gastro-intestinal absorption of lead in rats using the radio-lead technique of Kello & Kostial (1973).

Levels of lead exposure

Shields & Mitchell (1941) have drawn attention to the fact that many of the experiments exploring the effect of calcium, phosphorus and vitamin D on the metabolism of lead have involved the use of diets containing much higher concentrations of lead than would be of significance in practical nutrition. For

*It is pertinent to note that Knox (1973) has computed a correlation between the dietary intake of a number of nutrients and the standardized mortality ratios for a number of diseases. Calcium, animal protein and vitamin C showed a negative correlation, whereas fat and vitamin D both showed positive associations. The possibility that alterations in mortality might be made through relatively simple dietary manipulations was implied.

example, Shelling (1932) used rations containing 1·2% lead, Sobel *et al.* (1940) used rations with 0·80–0·82% lead, with much higher concentrations in their earlier work, Tompsett (1939) fed 1 mg lead to each of his mice daily, and Wittgens & Niederstadt (1955) used periodic surges totalling 2·3 g lead/kg body weight. Four studies, however, have used modest levels of lead exposure. Ardelean *et al.* (1955) used 0·04 g lead/kg body weight/week in their studies with rats, Lederer & Bing (1940) fed diets containing 0·01% lead, Grant *et al.* (1938) used diets varying in lead content from 0·0013 to 0·0512% and Barltrop & Khoo (1975) used diets containing 0·075% lead.

In an attempt to study the relationship between dietary calcium, phosphorus and lead at lead levels likely to be encountered in the environmental sense, Shields & Mitchell (1941) fed rats on diets containing 0·0015–0·0033% lead. At the termination of the feeding periods, the lead contents of the empty carcass, the bones and the soft tissues showed that a low content of calcium or of phosphorus, or of both, in the diet induced a high retention of lead by comparison with diet containing higher mineral levels. Indeed, the only method of securing lead storage in adult rats at this level of dietary lead was to lower the calcium content of the diet to inadequate or borderline levels. The precise level of dietary exposure cannot be established in the radio-lead study of Kello & Kostial (1973), involving a single oral dose of 2 μg lead chloride containing 10 μCi of ^{203}Pb, but it is clearly within typical environmental levels.

Although the total level of exposure to environmental lead cannot be defined for an individual, it is clear from public health studies that body burdens of lead can be related to the environment (Department of the Environment, 1974). At the levels of exposure likely to result from environmental contamination, it is important to note that processed milk products, which might be given prophylactically, can contain considerably higher concentrations of lead than fresh milk (Lamm, Cole, Glynn & Ullmann, 1973; Lamm & Rosen, 1974; Stelte, 1971) and that the young could be at special risk because of their substantially higher rate of gastro-intestinal absorption (Alexander, Delves & Clayton, 1973).

The highest levels of lead intake in young children are usually associated with pica and the highest incidence of pica overlaps that of lead poisoning, which occurs between the ages of 18 and 30 months (Lin-Fu, 1973). However, Sayre, Charney, Vostal & Pless (1974) question this perverted appetite for leaded paint as the main source of the elevated body burdens of lead found in inner-city children. These authors found lead on inner-city household surfaces and on the hands of children living in such houses at levels high enough to afford both a significant alternative source of exposure and a mechanism of ingestion.

The body burdens of lead of some inner-city children are likely to be further enhanced by inadequate calcium intake (Six & Goyer, 1970) and this risk may be compounded by the involvement of calcium deficiency in the production of lead pica (Snowdon & Sanderson, 1973). However, apart from reports that many children with lead pica drink large amounts of milk, there is no direct evidence that calcium deficiency enhances lead pica in humans.

In view of the growing acceptance that lead intoxication may take on forms different from those traditionally associated with clinical lead poisoning (David, Clark & Voeller, 1972; Seppäläinen & Hernberg, 1972) and that subtle but serious effects may be occurring at the soft-tissue levels of lead described by David *et al.* (1972), it is singularly unfortunate that cows' milk should enhance lead absorption when lead levels in soft tissue are diminished by a high absolute intake of approximately equal amounts of calcium and phosphorus.

Conclusions

The evidence reviewed here indicates that although milk contains components that hinder lead absorption, its overall effect is to promote the absorption of lead from the intestinal tract. However, the enhancement of lead absorption by milk may be a function of the intrinsic calcium and phosphorus requirements of the subject and the composition of the diet, especially the levels of iron and vitamin D. It is unfortunate that whole milk can no longer be considered as a prophylactic agent for urban children from poorer areas where there is a combined risk of malnutrition and high exposure to lead.

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Fd Cosmet. Toxicol. Vol. 13, p. 565. Pergamon Press 1975. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Toxicological Evaluation of Certain Food Additives with a Review of General Principles and of Specifications. Seventeenth Report of the Joint FAO/WHO Expert Committee on Food Additives. *Tech. Rep. Ser. Wld Hlth Org.* 1974, **539.** p. 40. Sw.fr. 5*.

Toxicological Evaluation of Some Food Additives Including Anticaking Agents, Antimicrobials, Antioxidants, Emulsifiers and Thickening Agents. Joint FAO/WHO Expert Committee on Food Additives. WHO/Food Add./74:5; *F.A.O. Nutr. Mtg Rep. Ser.* no. 53A, Rome, 1974. pp. xix + 520. Sw.fr. 23*.

These two publications are the result of the FAO/WHO Expert Committee's deliberations on a wide range of important food additives. The first-named volume summarizes the Committee's recommendations on acceptable daily intakes (ADIs) of these additives and on requirements for further toxicological and metabolic studies, as well as its conclusions on several general principles of safety evaluation, while the second gives details of the toxicological data on which the specific recommendations were based. The additives considered fell under the general headings of anticaking agents, antimicrobials, antioxidants and synergists, emulsifiers, thickening agents (including celluloses, vegetable gums, modified starches and dextrins, agar, alginates and carrageenan) and a miscellaneous group, which included mineral oil, monosodium glutamate, polyvinylpyrrolidone, propylene glycol and stearoyl lactylate.

The Committee has now abandoned its previous policy of allocating 'unconditional' and 'conditional' ADIs. For some of the compounds (e.g. benzoic acid, nitrates, sodium diacetate and thiodipropionic acid and its dilauryl ester), the conditional ADI was deleted, while for others (e.g. sorbic acid and its salts, ethylenediaminetetraacetate salts, tocopherols, diacetyltartaric and fatty acid esters of glycerol and polyglycerol esters of fatty acids), the previous conditional value was adopted as the ADI. Other changes included the lowering of previous ADIs for nitrites and hexamethylenetetramine, the latter because of indications of foetotoxicity in the dog, and an increase in the existing unconditional ADI for sulphur dioxide and sulphites on the basis of recent long-term and three-generation studies.

The first of the above reports also discusses the general problem of tissue uptake and storage of macromolecular food additives, noting that information on this topic is frequently lacking and recommending that further studies be conducted. Another problem considered is allergy to food additives, and it is stated that no approval would be given for the use of a substance causing serious or widespread hypersensitivity reactions. The practicability and effectiveness of label identification for lesser allergens is regarded as uncertain but as meriting further study.

The Committee judges the Council of Europe approach to flavourings (*Cited in F.C.T.* 1975, **13**, 459) to be useful and practical, although the Council's guide to toxicological testing and evaluation of flavourings is regarded as far too detailed and inflexible, in view of the fact that each compound "presents an individual and unique problem". It is also noted that the Council's lists are not comprehensive, and are unlikely to incorporate all substances used as flavourings. Some general principles for the acceptance of flavourings, with particular regard to the use of safe solvents and processes and the development of suitable specifications, are elaborated by the Committee.

Many of the food additive specifications prepared at previous meetings are considered to need revision. Attention is drawn to the possible presence of chlorinated dioxins in fatty acid derivatives and of ethylene and diethylene glycols and ethylene chlorohydrin in polyoxyethylene-containing esters. More information is required on the extent and level of these types of contamination. It is recommended that after revision all specifications should be published in a single compendium, with details of relevant analytical methods, test solutions and reagents.

*Obtainable in the UK through HMSO.

Fd Cosmet. Toxicol. Vol. 13, pp. 567–569. Pergamon Press 1975. Printed in Great Britain

BOOK REVIEWS

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 50. Edited by F. A. Gunther. Springer-Verlag. New York, 1974. pp. xi + 179. $20.00.

Nowadays the simultaneous or sequential application of herbicides, fungicides, insecticides and/or growth regulators to crops is a common agricultural practice. Problems that may be associated with this procedure are comprehensively reviewed in a chapter included in this volume of *Residue Reviews*. The review is concerned with the interaction of chemicals that, when applied in combination, elicit responses not predictable from the effect of each chemical applied singly. For example, several classes of herbicides may interact with organophosphate and carbamate insecticides. Frequently, increased toxicity results, although in some cases, such as the trifluralin-organophosphate interaction, antagonism occurs. It is deduced that the basis for herbicide-insecticide interaction is frequently related to alteration of the absorption or metabolism of one compound by the other. The review documents sufficient reports of pesticide interactions in plants to indicate that such interactions may be frequent contributors to the unpredictability of herbicidal effects. It warns that caution must be exercised in combining herbicides with insecticides and fungicides where the compounds have a potential for interaction. Crop injury could result, although on the other hand certain combinations at low application rates might produce the desired level of control with no crop injury and with less potential for residue accumulation in the soil.

The first part of a review on the analysis of pesticides by the thin-layer chromatographic–enzyme inhibition technique (TLC–EI) was published in a previous volume in this series (*Cited in F.C.T.* 1973, **11**, 1111). Part II has now made its appearance. This deals primarily with the application of TLC–EI to the analysis of pesticide residues and metabolites, but it also considers papers dealing with TLC–EI analysis of heavy metals, phosphatase inhibitors, chlorinated benzenes and phenolic compounds. An alphabetical list of organophosphorus and carbamate pesticides serves as a quick reference for the compounds that have been analysed by TLC–EI techniques and are mentioned in the review.

Last, but by no means least, Volume 50 contains various indexes. These include a cumulative subject index and a cumulative author index for Volumes 41–50 and a list identifying by their short titles all the papers published in each of Volumes 1–50.

Manual of Contact Dermatitis. By S. Fregert, on behalf of the International Contact Dermatitis Research Group. Munksgaard, Copenhagen, 1974. pp. 107. Dan. Kr. 48.00.

At best, this little book is a concise summary of what everyone needs to know about contact dermatitis and can't quite remember. The opening chapters briefly summarize the properties of the skin and the different types of reaction that can occur. Chapter 5 is a competent listing of the commonest contact allergens (sensitizers) and indicates where they are most likely to be encountered. In chapter 6, reactions to ingested allergens are dispatched in 15 lines. This seems a little terse, particularly as that popular example, tartrazine, has escaped mention. Further chapters cover irritant contact dermatitis and its commoner causative factors, and phototoxic and photoallergic contact dermatitis. A thorough description of the principles of patch testing, and guidelines on the detection of the cause of an allergy are given, and the book closes with short chapters on the prevention and treatment of dermatitis and the rehabilitation of dermatitis sufferers.

The text is well indexed and the layout is clear, but only a few general references are given. More thorough referencing would have been valuable, particularly as the book is said to be intended mainly for dermatologists who are not specialists in contact dermatitis and as a guide for use in postgraduate training in dermatology. The author has provided a ready handbook for those who need to check certain aspects of dermatitis diagnosis, but there are places where brevity and consequent oversimplification may be misleading. For example, it seems rash to say: "Thioglycolates in permanent waving solutions are not sensitising", even though cases of sensitization are extremely rare.

Perhaps in a second edition the International Contact Dermatitis Research Group could expand on what is essentially a very good idea.

Mycotoxins. Edited by I. F. H. Purchase. Elsevier Scientific Publishing Co., Amsterdam, 1974. pp. xiii + 443. $44.25.

Mycotoxins have been a growth area of research since aflatoxin was discovered in the early 1960s. As Dr. Purchase points out in his introduction, the proliferation of literature in this area is ever-increasing and highly complex, and a book this size cannot hope to cover all aspects of mycotoxicology. However, the 20 chapters range over a wide spectrum of fungal toxins, providing a comprehensive review of the data on some of the more common ones. Among those covered are aflatoxins, rubratoxins, ochratoxins, sporidesmin, trichothecenes and the range of diseases in which they are implicated, citreoviridin, luteoskyrin, slaframine, patulin, ipomeamarone and ergot, which was perhaps the first recognized mycotoxin.

Although most of the diseases attributed to fungal infection of foodstuffs have been observed in animals, the risks to man from individual toxins have been assessed wherever possible. Descriptions are given of the identification of toxins responsible for various diseases, including turkey X disease associated with afla-

toxin, the facial eczema caused in sheep by sporidesmin from *Pithomyces charterum* on their grazing land, slobbering in cows due to slaframine produced by *Rhizoctonia leguminicola* on red clover, and stachybotryotoxicosis caused by trichothecene derivatives resulting from infection of feed with a saprophytic mould, *Stachybotrys alternans*. As far as is possible, a common format has been used for each chapter. Information on the diseases produced and their geographical distribution, on the fungi involved and their taxonomy and life-cycles, on the toxins identified, including details of their biosynthesis, chemistry and analysis, and on animal toxicology is given in a logical and concise manner. Quite surprisingly, this approach to what could be a bewildering and somewhat arid subject, has produced an eminently readable text.

The volume is well referenced, printing errors seem to be few and far between, and the index appears to be quite thorough. Although the book is intended primarily for students, it should be a helpful source of information and reference for anyone with an interest in this field.

Birth Defects. Proceedings of the Fourth International Conference, Vienna, Austria, 2–8 September, 1973. Edited by A. G. Motulsky and W. Lenz. Excerpta Medica, Amsterdam, 1974. pp. xviii + 373. $40.50.

Congenital defects have attracted considerable attention in the last 15 years and the interest generated has led to the establishment of the new discipline of teratology. Much effort has been spent in describing the morphology of human and animal defects and in identifying their causes, and recently considerable attention has been paid to the experimental induction of birth defects by both physical and chemical agents, principally with a view to devising valid tests for the detection of teratogenic agents. The impressive advances that have been made in these fields and in the understanding of the subject at the molecular level occupied the majority of the communications presented at the 4th International Conference and reported in this book.

Many of the chapters give an account of the structure and function of genetic material. Despite the fundamental importance of this information to the understanding of the molecular basis of birth defects, it is not on the whole of immediate relevance to toxicologists. Another major section of the book is devoted to practical considerations dealing mainly with the diagnosis of birth defects *in utero*. These are of interest to the clinician who may have to decide on the termination of a pregnancy but again have little place in the practice of toxicology. In fact, the chapters of specific interest to those engaged in the safety evaluation of chemicals are only two. One of these, on the dominant lethal test, is written by W. Buselmaier, while the other, by H. V. Malling, considers the host-mediated assay. Unfortunately, these chapters are too short for the extensive ground covered and consequently tend to be superficial and uncritical in their consideration of many of the points raised.

Therefore, despite its excellent presentation, this book offers relatively little to the toxicologist. It will be of value chiefly to those interested either in unravelling the complex genetic background of birth defects or in the prenatal diagnosis of foetal malformation.

Perinatal Pharmacology: Problems and Priorities. Edited by J. Dancis and J. C. Hwang. Raven Press, Publishers, New York; North-Holland Publishing Co., Amsterdam, 1974. pp. xii + 228. $23.10.

This book presents the proceedings of a 3-day conference, held in Bethesda, Maryland, in April 1973, on existing problems and research priorities in perinatal pharmacology. The book comprises five main sections, dealing in turn with the effects of drugs on various aspects of foetal growth and differentiation, with the use of organ- and tissue-culture studies in this field, with the role of the placenta in mediating the effects of drugs on the foetus, with the influence of nutritional factors in perinatal pharmacology and with the effects of narcotics on the foetus. Each section reports the work of various contributors and at the end of each paper there is a list of references and, generally, a verbatim report of the discussion prompted by the presentation.

Common tendencies in published reports of conference proceedings are that the text may prove relatively lengthy for the amount of information presented and some of the discussions may border on the irrelevant or be unnecessarily argumentative, two disadvantages which this particular publication does not altogether avoid. Nevertheless, the lay-out is simple and the quality of presentation, including that of the figures and tables, is good. The book ends with an adequate, although not exhaustive, index.

Before this, there is a final contribution entitled "Research Goals in Developmental Pharmacology". This was presented by Dr. B. L. Mirkin and warrants particular comment. Following his assertion that because "the concerns of developmental pharmacology may range from an assessment of drug influences at the time of administration to their effects at the completion of biologic maturation, a panoramic perspective is a necessary prerequisite for investigations in this area", the author lists, at great length and with many sub-divisions, the questions in this field requiring further investigation. This somewhat unusual approach, backed by Dr. Mirkin's experience as Chairman of an NAS-NRC committee studying this problem, merits a great deal of thought. The penultimate paragraph of this paper is a plea for greater leadership from the appropriate governmental health agencies in nurturing the discipline of developmental pharmacology—a plea, in effect, for more money.

In spite of this excellent final chapter, with its detailed consideration of experiments necessary to further the subject, the proceedings as a whole suggest that a great deal of work, much of high quality, is already being carried out in this field, yet relatively little attention is being paid to relating this to the problems encountered in the unborn or newborn human infant. This is the greatest and most difficult challenge to meet.

This is a very readable book, and should prove well worth while if only to stimulate others into carrying further some of the ideas suggested by Dr. Mirkin.

La Réglementation des Produits Alimentaires et Non Alimentaires. Répression des Fraudes et Contrôle de la Qualité. By R. A. Dehove. 8th ed. Commerce–Éditions, Paris, 1974. pp. xii + 1154. 165 fr.

In France, the Constitutional Law of 1905 forms the basis for the control of the sale of all merchandise, including food and drink, and has given rise to a complex system of decrees, directives and official circulars designed in various ways to protect the consumer. The publication named above draws together all those items of legislation concerned with consumer protection from the viewpoints of both public health and fraudulent practice, identifying in all cases the relevant decrees and directives for those who need to consult the original texts. The publication has been up-dated at regular intervals since it first appeared in 1954, and the eighth edition incorporates in chronological order amendments published after the preceding edition was produced in 1971, including some items of 1974 legislation.

In format the eighth edition closely resembles the seventh, which was reviewed some time ago (*Cited in F.C.T.* 1972, **10**, 564). The major part of the publication is again taken up with details of established food standards, and—as before—there are chapters dealing with food hygiene, labelling requirements, permitted food additives, animal feeds, agricultural chemicals and household products. There have been important developments in several of these fields since the appearance of the last edition, notably in connexion with materials and objects intended for use in contact with food. Details of steps taken within the framework of the Common Market towards harmonization of legislation in the Member States are included in the edition under review. Another useful inclusion is an indication, where possible, of information on established use, a helpful adjunct to the "good manufacturing practice" concept frequently provided as a guideline where no specific regulations have been laid down. Finally, for ease of reference, there is a comprehensive subject index.

Those concerned with these French regulations will be grateful for this latest version of an extremely useful guide. Long may subsequent editions of "Dehove" continue to appear!

BOOKS RECEIVED FOR REVIEW

The Normal Microbial Flora of Man. The Society for Applied Bacteriology Symposium Series No. 3. Edited by F. A. Skinner and J. G. Carr. Academic Press, London, 1974. pp. xv + 264. £6.00.

Connective Tissues. Biochemistry and Pathophysiology. Edited by R. Fricke and F. Hartmann. Springer-Verlag, Berlin, 1974. pp. xii + 309. $20.00.

Carcinogenesis as a Biological Problem. By I. Berenblum. North-Holland Publishing Company, Amsterdam, 1974. pp. xxvii + 376. Dfl. 90.00.

Practical Methods in Electron Microscopy. Vol. 3. Edited by Audrey M. Glauert. North-Holland Publishing Company, Amsterdam, 1974. pp. xvi + 353. Dfl. 90.00.

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Information Section

ARTICLES OF GENERAL INTEREST

LITTLE MORE ON THE FRYING FRONT

It has been recognized for many years that certain oxidized and heated fats liable to be present in human and other diets are toxic to experimental animals and may retard growth or induce pathological tissue changes (*Cited in F.C.T.* 1968, **6**, 653). In some instances polymerization occurs, and the heating process may promote several other types of chemical reaction; some of the toxic substances produced have shown indications of carcinogenic potential (*ibid* 1966, **4**, 344).

The *British Medical Journal* (1974, **3**, 338) has recently commented that the commonly used cooking oils (peanut, olive, rapeseed, soya-bean, sunflower, maize and cottonseed) contain, in the raw state, polycyclic aromatic hydrocarbons and sometimes aflatoxins (as does peanut oil made from infected nuts). Refining processes eliminate all the aflatoxin and most of the hydrocarbons. Tumour-like stomach lesions, which have been observed in experimental rodents fed a high-oil diet, are more likely to be the result of dietary deficiencies, in particular a lack of vitamin A. The latter deficiency frequently occurs in animals fed diets rich in unheated fats and may interfere with the healthy development of mucosal tissue.

Heating as a factor

The desiderata governing the use of frying fats have been given by Lang (*Z. ErnährWiss.* 1972, **11**, 177). They include the choice of an unobjectionable frying fat or oil, the taking of precautions to avoid heating at a temperature exceeding 180°C and against local overheating due to the use of faulty equipment and techniques, and the general avoidance of prolonged heating. However, the content of polycyclic aromatic hydrocarbons, including some potential carcinogens, actually falls as a result of heating, when compared with that of the unheated material. Lang *et al.* (*ibid* 1973, **12**, 241) have recorded the results of feeding rats over two generations with a diet containing 10% partially hydrogenated peanut oil or soya-bean oil heated in each case at about 175°C for up to 96 hours, alone or with frying foodstuffs. There was no significant difference in lifespan between the groups fed unheated and heated oils, and the presence of other food during the heating process made no material difference. There was a tendency for mortality to be increased during the early stage of feeding with unheated oils, but this became significant only in one experimental group, that of first-generation rats given unheated soya-bean oil. Nevertheless, Lang (*Fette Seifen AnstrMittel* 1973, **75**, 73) has pointed out that the regular consumption of overheated fats impairs the health of experimental animals to a degree that correlates with the degree of overheating. Properly heated fats, on the other hand, have shown a reduced content of carcinogenic polycyclic hydrocarbons and a correspondingly lowered tumour-inducing capacity, together with an induction of hepatic microsomal-enzyme activity, when given to rats in a three-generation feeding study (*idem, ibid* 1974, **76**, 145).

The harmlessness of properly heated fats has been given further support by Nolen (*J. Nutr.* 1973, **103**, 1248), who fed dogs on diets containing 15% fresh, partially hydrogenated soya-bean oil or 15% of a similar oil previously used for the commercial deep-fat frying of potatoes, scallops and onion rings at 182°C to a point when excessive foaming occurred and the oil was considered unsuitable for further frying. Male dogs gained more weight on the fresh-fat diet than on the heated one, but similar variations were seen in individual dogs on a commercial diet. Only one female showed a slight reduction of growth while taking fresh fat, the others showing a slightly higher gain than those on the used-fat or commercial diets. These findings were probably due to a slightly lower absorption of used than of fresh fat by the males, a difference that was not apparent in the females. The groups did not differ in respect of serum cholesterol, phospholipid and glucose after 11 and 44 weeks of feeding. Clinical observation revealed no adverse effects and there were no abnormalities on histological examination of the organs.

Rao *et al.* (*Lipids* 1973, **8**, 342) have reported a decrease in acetate metabolism in the livers of rats fed heated corn oil in diets containing 10–30% levels of protein compared with the rate in rats on similar diets containing fresh corn oil. The heated oil had been maintained at 190°C for 132 hours. At the end of the 18-week feeding period, each animal was given an ip injection of sodium [1-^{14}C]acetate and radioactivity was measured at different times in the exhaled air and in the lipid fraction of liver. In the 10%-protein group, 50% of administered ^{14}C appeared in the expired air within 50 minutes when fresh oil had been fed, but only after 90 minutes in rats given heated oil. Recovery of $^{14}CO_2$ was slower in the 20%- and 30%-protein groups than in the 10% groups; times for recovery of 50% of administered radioactivity after consumption of fresh and heated oils were 100 and 110 minutes, respectively, in the 20%-protein group and 130 and 140 minutes in the 30% group. Cellulose supplementation of the 10%-protein diet increased the rate of acetate metabolism in rats fed heated fats, the 50% recovery time being reduced to 70 minutes. The decreased rate of acetate metabolism was reflected in a higher lipid content, mainly of unsaturated triglyceride, in the livers of rats fed the heated oil. The monoene, diene and triene fractions of liver lipid carried nearly twice as much radioactivity in animals fed heated oil than in those fed fresh oil.

Oxidation as a factor

Oxidized fats in the diet may have undesirable effects on lipoprotein synthesis. Privett & Cortesi (*ibid* 1972, **7**, 780) raised rats on a sucrose–casein diet containing the necessary vitamins and minerals and supplemented with 10% safflower oil, menhaden oil or hydrogenated coconut oil, or with no fat at all. Safflower and menhaden oils were also fed in oxidized form after their exposure to air in the dark at room temperature for 2–8 days. The oxidation process reduced the linoleic acid content of safflower oil but did not otherwise change its fatty acid composition markedly. On the other hand, menhaden oil showed marked changes in polyunsaturated fatty-acid composition when oxidized. Feeding of both oxidized oils depressed the growth of the rats, increased the heart and lung weights in relation to body weight, and decreased the relative weight of epididymal fat pads and vesicular glands. Red-cell haemolysis did not occur in animals fed an oxidized-oil diet not deficient in vitamin E, or a fat-free diet lacking the vitamin, but when tocopherol acetate was omitted from the vitamin and mineral supplements, oxidized-oil diets induced fragility of the membrane of both erythrocytes and liver mitochondria. It is evident therefore that vitamin E has a protective effect against the adverse effects of oxidized oils.

Kaunitz & Johnson (*ibid* 1973, **8**, 329) have described the feeding of rats with cottonseed, olive, corn, soya-bean and coconut oils, chicken fat, beef fat, butter oil, lard and saturated medium-chain triglycerides, both in the fresh state and after aeration at 60°C for 40 hours. Body weight and lifespan varied according to the type of fat fed, but not in relation to its aeration or otherwise. Cardiac damage, mainly focal myocarditis and fibrosis, was most marked in animals fed corn oil, followed in descending order of myocardial toxicity by cottonseed, soya-bean and olive oils, beef fat, saturated medium-chain triglycerides, butter oil, chicken fat and lard. Aeration increased the incidence of myocardial lesions produced by cottonseed, soya-bean and olive oils, beef fat, chicken fat and lard, but did not alter the effect of corn oil or medium-chain triglycerides. Cellular damage in heart-muscle fibrils was indicated by their increased resistance to staining with Light Green SF Yellowish. In addition to the heart lesions, oxidized vegetable fats (but not animal fats) induced a higher incidence of severe bile-duct proliferation in the liver than did the corresponding unoxidized fats. The mild changes brought about in the fatty-acid content of the oils by aeration oxidation seem unlikely to account for the noted increases in toxicity, and the suggestion is made that pharmacologically active compounds present in the non-triglyceride fraction of the fats, or some currently unsuspected environmental contaminant, may be markedly altered by oxidation and may be responsible for the lesions.

[P. Cooper—BIBRA]

THE FATE OF FLUOROCARBONS, INHALED OR INGESTED

Much has been stated, argued and implied about the safety of fluorocarbon propellants in pressurized aerosols. Since the report of an epidemic of sudden sniffing deaths in 1970 (*Cited in F.C.T.* 1971, **9**, 730), numerous workers have devoted their attention to the problem, with all kinds of novel experimentation (*ibid* 1974, **12**, 552; *ibid* 1975, **13**, 393) and have succeeded in demonstrating in a variety of ways that fluorocarbons can cause cardiac sensitization in certain animal species. Concern has therefore been expressed also about the safety of bronchodilators given by inhalation. During this flurry of activity, a still small voice of calm in the UK commented on the need to compare blood levels of fluorocarbon in animals during provocation of cardiac sensitization with those in man after the use of bronchodilator aerosols (*ibid* 1971, **9**, 751). It seems that threshold levels of both fluorocarbon and adrenaline in blood (or tissue) are required for the heart to become sensitized. This question of the correlation between levels causing an effect in animals and levels to which man is exposed has received only limited attention until fairly recently.

In a previous look at fluorocarbons, we referred to an investigation into human blood levels of fluorocarbon after the use of bronchodilator aerosols (*ibid* 1975, **13**, 396). Two papers have since presented comparable data for animals. Azar *et al.* (*Am. ind. Hyg. Ass. J.* 1973, **34**, 102) and Trochimowicz *et al.* (*ibid* 1974, **35**, 632) have investigated the blood levels of various fluorocarbons in beagle dogs during 10-minute exposures to concentrations known to cause cardiac sensitization. Their method consisted essentially of exposing cannulated, unanaesthetized animals to the propellants by means of a face-mask and taking arterial and venous blood samples at frequent intervals between 0 and 25 minutes after the start of the exposure. The fluorocarbon content of the blood was determined using gas chromatography with electron-capture detection. A fairly wide variation in blood levels between the individual animals probably reflected differences in the rate and depth of respiration. However, the levels in all animals tended to follow a similar pattern of change with time. Blood levels rose rapidly during the first 5 minutes of exposure and then levelled off, declining sharply at the end of the exposure and subsequently showing a slower rate of disappearance. Fluorocarbon levels were higher in arterial than in venous blood during exposure and lower following termination of the fluorocarbon treatment. This finding has also been observed with anaesthetic gases and indicates an initial uptake of fluorocarbon by the tissues and subsequent release of the compound when exposure is terminated.

An interesting finding was that although the concentrations of fluorocarbons required to produce cardiac sensitization vary widely, the blood levels resulting from exposure to these concentrations differed

little among the C_2 fluorocarbons studied or between the two C_1 compounds:

Fluorocarbon	Exposure concn (vol %)	No. of dogs sensitized	Blood concentrations (μg/ml)	
			Arterial	Venous
F11 (CCl_3F)	0·5	1/12	28·6	19·7
F12 (CCl_2F_2)	5·0	5/12	35·3	22·8
F113 ($C_2Cl_3F_3$)	0·5	10/29	12·5	4·9
F114 ($C_2Cl_2F_4$)	2·5	1/12	13·8	7·2
F115 (C_2ClF_5)	15·0	1/13	5·8	3·9

This may reflect differences in blood solubility, although there is no apparent reason on physical grounds for the finding that the C_2 fluorocarbons sensitize the beagle heart at lower blood concentrations than their C_1 counterparts.

The group responsible for a recent study on blood concentrations of F11 and F12 in man following the use of a proprietary bronchodilator aerosol (*Cited in F.C.T.* 1975, **13**, 396) has now taken a look at the F11 and F12 content of alveolar air after exposure to both metered and breath-actuated aerosols (Draffan *et al. Thorax* 1974, **29**, 95). Mass spectrometry was used to measure F11 and F12 levels in successive expired breaths for up to 2 minutes after the inhalation. The mass spectrometer was standardized against a gas chromatograph using a known sample of F11 and F12. Six healthy adult volunteers and two patients with obstructive airway disease inhaled from a placebo aerosol containing 28·7 mg F11 and 41·0 mg F12/metered dose. The breath was held for 5 seconds and then the subjects breathed normally through a mouthpiece connected to the mass-spectrometer system. The propellants were measured on alternate breaths for 30–120 seconds. Similar procedures were carried out with a further seven subjects using a breath-actuated aerosol releasing a 1:2:1 (w/w) mixture of F11, F12 and F114 in a dose of 25 μl/valve actuation. In this case, F11 and F12 were not analysed individually because of the superimposed spectrum of F114. Alveolar concentrations of fluorocarbon were measured in another volunteer after repeated inhalation of the placebo aerosol on successive breaths for 30, 60 or 120 seconds (6, 12 and 24 doses). The subject's electrocardiogram was recorded throughout the latter study.

Two patterns of fluorocarbon exhalation were seen with the conventional metered-dose aerosol. In the first, there was a short initial lag while the dead space was cleared, and then a rapid rise to a plateau representing the alveolar fluorocarbon. The second pattern consisted of an immediate high spike followed by decreasing levels of fluorocarbon and resulted from late firing of the aerosol and consequent retention of most of the propellant in the buccal cavity and upper dead space. The latter type of result, which was disregarded for the purposes of the experiment, was eliminated by the use of breath-actuated aerosols. The peak alveolar-gas concentrations determined for any one individual appeared to be reasonably reproducible but variation between subjects was somewhat greater, ranging from 1·6 to 4·8 μg/ml for F11 and from 4·2 to 7·9 μl/ml for F12. The levels found were somewhat lower than those predicted, particularly in the case of F11, probably on account of uptake into the blood and lung tissue, the blood/gas partition coefficients for F11 and F12 being 0·9 and 0·25, respectively.

The mean levels of 2·7 and 5·5 μg/ml demonstrated for F11 and F12 in alveolar air after a single discharge of the inhaler were only 4·0 and 1·4%, respectively, of those that, when maintained for 5 minutes in dogs, sensitized the myocardium to adrenaline-provoked ventricular arrhythmia. In these human studies, the elimination half-life of the fluorocarbons averaged 13·7 seconds for F11 and 14·8 seconds for F12, the maximum being 29·7 seconds for F12 in one subject. Comparable results were obtained in the two patients with destructive disease of the airways, and no hazard can be envisaged from the normal use of a pressurized medical aerosol unless the level required to sensitize the heart is very much lower in man than in dogs.

Higher peak alveolar concentrations of propellant were achieved on repeated inhalation. The fact that values after 24 doses were only slightly higher than those after 12 suggests that these values of 29·6 and 26·1 μg F11/ml and 66·9 and 65·0 μg F12/ml, respectively, may represent the highest obtainable by this mode of administration. Above this level the amount inhaled is similar to the amount exhaled. The peak concentrations after repeated inhalation approached those that sensitize the dog heart to arrhythmia, although the electrocardiogram remained normal in the subject under test. Thus, actuation of the inhaler on every breath might lead to an untoward response, and instructions provided with inhalers now warn patients of the risks of overdosage.

Although the elimination half-lives found in this set of experiments were very short, it has been suggested that the short half-life of a fluorocarbon in blood is due to its rapid distribution in the tissues and that the actual biological half-life of the compound may be longer than it appears (*Cited in F.C.T.* 1975, **13**, 396). The group responsible for the above study has also looked into the fate of labelled F11 after inhalation in both man and the rat (Williams *et al. Thorax* 1974, **29**, 99). F11 labelled with fluorine-18 ([^{18}F]F11) was prepared from cyclotron-irradiated silver fluoride. Anaesthetized rats, exposed to 0·3% (v/v) [^{18}F]F11 in air in a sealed box for 6 minutes, were killed 0, 15 or 60 minutes after exposure and [^{18}F]F11 was measured in whole blood, heart muscle, skeletal (psoas) muscle, liver, kidney, spleen, lung, brain, adrenal and fat. In estimating the whole-body [^{18}F]F11, a correction was made for adsorption on to the skin and fur, a contribution found to be less than 1%. High concentrations of [^{18}F]F11 found at 0 minutes (i.e. in rats killed before removal from the fluorocarbon atmosphere) decreased rapidly in the blood, heart and other tissues with a high rate of blood flow once the animals started breathing air. Elimination from fat was very much slower and had reached only slightly less than half its high initial value within 1 hour. Levels in the adrenals were also high initially and at 15 minutes, being fairly close to those in the fat at these times, but by 60 minutes the adrenal concentration was comparable with the low levels in the other organs.

The fate of [^{18}F]F11 was also examined in man. Four healthy volunteers inhaled one metered dose of fluorocarbon and held their breath for 5 seconds, after which they breathed normally. The exhaled air was

removed by exhaust ventilation. The [^{18}F]F11 absorbed was traced by means of a gamma camera placed against the body and an NaI (Tl) detector placed 2 m away to carry out whole-body counts. Whole-body measurements were taken before and for 20 minutes after fluorocarbon inhalation. Pictures from the gamma camera, recorded for 2 minutes immediately after inhalation, were processed on a CDC 6600 computer to give activity/time curves for various areas of the body. As in the rat, the whole-body count fell rapidly in the first 2 minutes. Again, transference of the fluorocarbon from the lungs to the blood stream and from there to the tissues was apparent, and was followed during a second phase by slower pulmonary excretion of fluorocarbon released from the tissues and fat. Fluorocarbon droplets did not appear to be deposited in the mouth during use of the aerosol.

The main factor affecting the fate of fluorocarbons in both rat and man appears to be the body fat. Fluorocarbons are concentrated in the fat and then slowly released into the blood at concentrations that should not cause any risk of cardiac sensitization. A further point noted by Williams *et al.* (*loc. cit.*) was the comparatively high concentration reached in the adrenals in both species, a finding that is presumably a reflection of the gland's lipid content. Perfluoro-*n*-hexane, a completely fluorinated compound (C_nF_{12}), has been found to inhibit microsomal oxidation, and the authors surmise that F11 may have similar effects. They point out, however, that high concentrations would be unlikely to persist long enough to have any effect on the formation of adrenal steroids.

These papers suggest that under conditions of normal use fluorocarbon propellants are not encountered in sufficiently high or persistent concentrations to cause adverse effects in man. The concentrations required to produce cardiac sensitization in animals can only be approached in man by repeated inhalation of metered doses from a pressurized aerosol (at a rate of at least 12–24 doses in 2 minutes). Another potential source of fluorocarbon has to be considered, however, now that foods qualify for pressurized packs, since the possibility of fluorocarbon ingestion may raise further questions.

Clayton *et al.* (*Am. ind. Hyg. Ass. J.* 1966, **27**, 234) carried out a 90-day inhalation study in dogs exposed to up to 10% (v/v) F115. At that time it was not envisaged that the primary use of F115 would be in food-packaging, but as this is now the case, Terrill (*ibid* 1974, **35**, 269) has examined the relationship between blood levels of F115 and its route of administration, in order to establish whether data obtained following inhalation could be considered applicable to the situation after ingestion.

Beagle dogs inhaled 10% F115 for 10 minutes or 6 hours or were given 8% (w/w) F115 in 150 g whipped topping (a fluorocarbon dose of about 1 g/kg) by gastric intubation. The right carotid artery and the right jugular vein were catheterized for blood sampling. The 10-minute exposures were carried out with a mask designed to enable delivery of a consistent fluorocarbon mixture and sampling of the exhaled gases, while the 6-hour exposure took place in a 1-m^3 chamber. The fluorocarbon content of arterial and venous blood and of exhaled air was determined by gas chromatography, the method being sensitive to 0·06 μg/ml.

In both inhalation tests, the blood levels of F115 rose rapidly at the start of exposure and reached a steady state within 5 minutes. There was little difference in the arterial decay rates after the 10-minute and 6-hour exposures, but the decrease in venous level was slightly slower after the 6-hour than after the 10-minute inhalation. However, the half-life for venous fluorocarbon loss following cessation of exposure was less than 5 minutes in all cases. Average arterial and venous blood levels of F115 were 2·9 and 2·1 μg/ml, respectively, during the 10-minute exposure, and blood levels were almost consistently below the limit of detection (0·06 μg/ml) throughout the 24 hours following oral administration of a single dose of 12 g F115. The exceptions were occasional samples of venous blood which were taken during the first 30 minutes after treatment and showed levels slightly above 0·06 μg/ml, but it was thought that other factors, such as sampling errors or inhalation of regurgitated gases may have been responsible for these results.

Thus absorption after ingestion is some 35–48 times lower than that after inhalation of a fluorocarbon. Currently available pressurized food packs contain about 500 μg F115/g food, suggesting that a person would have to eat more than his own weight of food in order to achieve blood levels equivalent to those resulting from inhalation of 10% F115 for 10 minutes. This will be reassuring for those who may be concerned about a possible visitation of cardiac arrhythmia as a result of the consumption of excessive amounts of cream topping!

[F. A. Charlesworth—BIBRA]

LIVING WITH POLYCHLORINATED BIPHENYLS

The widespread occurrence of polychlorinated biphenyls (PCBs) throughout the environment, their relatively high toxicity and their exceptional resistance to biodegradation have all served to draw attention to these unwelcome companions and to dictate a careful watch on their progress in natural ecosystems. We have already taken a broad view of the challenge posed by PCBs and have noted that the prompt action of the Monsanto group, the sole manufacturer in the USA and UK, in restricting the use of PCBs to closed systems such as transformers and capacitors, has alleviated the natural anxiety of toxicologists and ecologists (*Cited in F.C.T.* 1973, **11**, 131). About this time, Maugh (*Science, N.Y.* 1972, **178**, 388)

summarized reports of the distribution of PCBs in the biosphere. They had appeared in the tissues of polar bears and seals in Canada, in 54·3% of 2189 samples of human adipose tissue collected throughout the USA. in nearly 20% of some 3500 samples of fish, milk, eggs and cheese investiaged by the FDA, and (at levels as high as 3·2 ppm) in the bottom sediments of lakes, which were, in some cases, far from likely industrial sources.

Concentrations of about 1 ppm seem to be generally accepted as offering no toxic hazard to man, and indeed the FDA has adopted interim tolerance limits for foods ranging from 0·2 ppm in infant foods to 5 ppm in the edible parts of fish (*Federal Register* 1974, **39**, 42747). There is, however, no guarantee that other animal species can withstand similar concentrations of PCBs without harm. Mink farmers have had reason to deplore the high susceptibility of their animals to the levels of PCBs found in some types of fish from the Great Lakes, while some PCBs (32–54% chlorine) adversely affect hatchability in chicks. Many planktonic organisms, which play an essential role in the food chains of both fresh- and salt-water fish, may be susceptible to low concentrations of PCBs and may serve to concentrate them in the food chain (Maugh, *loc. cit.*).

Occurrence of PCBs

Finklea *et al.* (*Am. J. publ. Hlth* 1972, **62,** 645) has remarked that PCBs of industrial importance are complicated mixtures of many of the 210 theoretically possible halogenated biphenyls plus possible contaminants. They have been used in synthetic rubbers, paints, plastics, insulating materials, caulking materials, flame retardants, cutting oils, synergists for pesticides, adhesives, sealants, printing inks, duplicating papers and heat-transfer media, uses for which their high boiling point, low water-solubility, miscibility with organic solvents, high dielectric constant, thermal stability and high resistance to chemical and biological degradation render them particularly suitable. Many of these properties, however, also ensure their place among the serious and persistent environmental pollutants. Thermal destruction, for example, occurs only above about 950°C, which means that waste oils burnt at ordinary furnace temperatures, and solid wastes containing PCBs, form a ready source of atmospheric contamination (Jensen, *Ambio* 1972, **1,** 123).

Maugh (*Science, N.Y.* 1973, **180,** 578) has reported that PCBs can be produced by irradiation of DDT vapour with ultraviolet light of wavelengths present in sunlight in the lower atmosphere (290–310 nm). This throws new light on the problem of contamination with other organochlorine compounds, a situation which for so long led to the overlooking of PCBs in analyses of a wide variety of samples from the environment. It may also help to explain the discrepancy between, on the one hand, the degree of accumulation of DDT and DDE to be expected in the biosphere from the known use levels of DDT and its assumed resistance to atmospheric degradation and, on the other, the amounts that can actually be accounted for by current methods of monitoring and calculation. DDE is an intermediate in the photodegradation of DDT to PCBs. Fortunately, photodegradation may be expected to increase environmental levels of PCBs only in respect of the less chlorinated homologues, which are probably less troublesome in biological systems and less persistent. Sewage treatment is a further source of PCBs, significant quantities of which were found in the effluent from a plant which took in waste from a textile mill using biphenyl as a dye carrier (Gaffney, *ibid* 1974, **183,** 367). This waste was treated with chlorine, and a suspension of filter scrapings was found to contain 18 ppm PCBs, which interfered with normal sewage-treatment processes.

PCBs in human tissues and milk

In a study of human perirenal fatty tissue obtained from 32 post-mortem samples taken in the Vienna district, Pesendorfer *et al.* (*Wien. klin. Wschr.* 1973, **85,** 218) found a mean concentration of 3·5 ppm PCBs, ranging from 0·3 ppm in a woman aged 81 to 8·5 ppm in a male aged 76. Associated with the PCBs was a mean fatty tissue content of 5·8 ppm total DDT and DDE. In view of the fact that, when body fat is consumed, stored organochlorine derivatives are liberated into the circulation, an investigation by Hesselberg & Scherr (*Bull. env. contam. & Toxicol.* (*U.S.*) 1974, **11,** 202) is relevant. Nine patients suffering from severe wasting disease were studied and compared with apparently normal subjects. DDE residues in the blood were much higher in these patients than in the controls, the mean levels for the two groups being 21 and 3 ppb ($b = 10^9$), respectively. The cachectic patients also showed a mean blood concentration of 47 ppb PCBs, which were not detectable in the controls. The authors dismiss the DDE residues in the cachectic patients as being less than those found among apparently healthy workers in a DDT plant, but they suggest that the levels of circulating PCBs may have been high enough to be harmful, although the patients' multiple symptoms would probably have masked any such effects.

In contrast to the figure reported from Vienna, a survey of human fatty-tissue samples analysed in Japan (Curley *et al. Nature, Lond.* 1973, **242,** 338) showed that while all the tissues analysed were positive for PCBs, the level in 80% of the samples was less than 1 ppm and the overall range was 0·3–1·48 ppm. The tissue levels of PCBs in ordinary persons in Japan and in others suffering from 'Yusho' disease, attributed to the consumption of rice oil contaminated with PCBs, have been compared by Masuda *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1974, **11,** 213). The average concentration of PCBs in the fatty tissue of the Yusho patients was 2·5 ppm (6·3 ppm in isolated fat) compared with 0·9 ppm (2·6 ppm in fat) in the controls. The livers of Yusho patients contained 0·1 ppm PCBs (6·5 ppm in fat). The gas-chromatographic (GLC) patterns of the tissue samples and of the PCBs present in the rice oil suggest that while the overall PCB levels decreased substantially during the year following the accident, this largely reflected a decrease in the PCBs of lower chlorine content; retention of the hexa- and heptachlorobiphenyls was more prolonged, apparently for as much as 4 years or more.

The long retention of PCBs in body fat raises the question of how much may be excreted into breast

milk and passed on to the infant. Masuda *et al.* (*loc. cit.*) reported an average of 0·014 ppm and a maximum of 0·02 ppm PCBs in milk from 12 normal Japanese women; milk samples from another group of 19 normal women showed an average level of 0·03 ppm and a range of 0·01–0·06 ppm. The sample available from the one appropriate Yusho patient also contained 0·06 ppm PCBs and gave a GLC pattern similar to residues from the liver and adipose tissues of the patients. Siyali (*Med. J. Aust.* 1973, **2**, 815) detected DDT and other common pesticide residues in the milk of 45 women in a metropolitan area of New South Wales but did not find any PCBs, and suggested, therefore, that the human food chain in that region was possibly not contaminated by PCBs. Savage *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1973, **9**, 222) reported low concentrations of PCBs in milk from some of a group of 39 women in rural Colorado. Only eight of the samples contained PCBs, six at a level of 0·04 ppm, one at 0·05 ppm and one, from a 25-year-old woman who had spent her entire life in Colorado, at 0·1 ppm.

Routes of entry into the food chain

Seas and lakes are known to contain low concentrations of PCBs. In a study of North Atlantic waters, Harvey *et al.* (*Science, N.Y.* 1973, **180**, 643) found that open surface water contained a mean concentration of 35 ng/litre, while water from 200 m down contained 10 ng/litre. The surface waters of the Sargasso Sea showed slightly lower concentrations (27 ng/litre) but there was no observed relationship between PCB concentration and the proximity of land masses, and it seems that the atmosphere must be the predominant mode of transport of these contaminants. The same group of workers recently published some encouraging data derived from subsequent studies of North Atlantic surface waters (*idem. Nature, Lond.* 1974, **252**, 387). These results indicated that by the end of 1973 the levels of PCBs in these waters were some 40 times lower than those found in 1972. In the Sargasso Sea, a rapid decline in concentration to 1 ng/litre in April 1973 had levelled off at 0·8 ng/litre by the end of the year. These reductions in surface-water concentrations roughly coincided with a tenfold decrease in PCB contamination of the atmosphere over the North-west Atlantic during 1973 (Harvey & Steinhauer, *Atmos. Environ.* 1974, **8**, 777) and a fivefold decrease in the PCB content of mixed plankton from this area between 1972 and September 1973 (Harvey *et al. J. mar. Res.* 1974, **32**, 103).

Ware & Addison (*Nature, Lond.* 1973, **246**, 519) have described how the concentration of PCBs in plankton from the Gulf of St. Lawrence varies with the mean size of the plankton species and the time of year. PCB concentrations were inversely proportional to particle size, even with a 34-fold difference in residue levels between composite plankton samples, and the acquisition of organochlorine contaminants from the environment therefore appears to bear a direct relationship to the surface area of the particles. Plankton particles in the 73–202 μm group contained what was thought to be the highest PCB concentration ever reported for natural plankton, namely 31 ppm (wet weight) in June. Overall PCB concentrations in the plankton varied greatly, from 93×10^{-8} g/m^3 at the beginning of June to 2×10^{-8} g/m^3 at the beginning of August, showing an irregular incremental pattern indicating that PCBs entered the plankton in discrete pulses, largely from the air and rainwater. This explanation was supported by the observed correlation between PCB contamination and the cumulative rainfall during the 10–20 days preceding the sampling. During periods of light precipitation the clearance of PCBs by small-plankton biomasses would account for the low PCB residues afterwards. Ware & Addison (*loc. cit.*) were careful to eliminate the interfering effect of PCBs in the antifouling paint on the hulls of their sampling vessels by checking the water of the wake. Jensen *et al.* (*ibid* 1972, **240**, 358) had previously reported that in sampling plankton in Stockholm harbour they found that samples from the wake could be heavily contaminated with PCBs and other lipophilic organochlorine compounds derived from the bottom paint. It seems likely that inshore water may regularly be contaminated with PCBs from shipping in this way, and that plankton may thereby acquire high concentrations of PCBs.

The effect of PCBs on plankton communities was described by Moore & Harriss (*ibid* 1972, **240**, 356), who studied the effects on photosynthesis of different concentrations of PCBs added to heterogeneous plankton samples taken from the Gulf of Mexico. Radiocarbon uptake by the samples was more depressed by the PCB mixtures, Aroclor 1242 and 1254, than by equivalent amounts of *p,p'*-DDT or pure 2,4'-dichlorobiphenyl. Inhibition indicating toxicity was evident with 1–2 ppb in the case of the Aroclors, whereas DDT and 2,4'-dichlorobiphenyl caused comparable inhibition at 5 and 7 ppb respectively. These effects on total plankton communities were more marked than those previously observed with isolated laboratory cultures of selected species.

From plankton, PCBs enter fish and seabirds. As regards marine mammals, Saschenbrecker (*Can. J. comp. Med.* 1973, **37**, 203) has remarked that it is not known whether whales ingest PCBs in their planktonic diet or absorb them directly from the water; but widespread oil pollution of the North Atlantic may produce an enriched surface stratum from which lipid-soluble contaminants, including PCBs, might be absorbed. Blubber tissue from 12 North American fin-back whales, collected in 1970 and 1971, showed a maximum of 0·185 ppm PCBs, compared with levels of DDT metabolites of up to 2·557 ppm.

A large proportion of the published observations seems to have been made on freshwater fish in the lakes of North America. Bache *et al.* (*Science, N.Y.* 1972, **177**, 1191) have reported that trout from Cayuga Lake in New York State showed an overall rise in PCB levels with age and maturity, the concentrations found ranging from 0·5 ppm in juvenile fish to 12–30 ppm in adults (8–12 years old). The presence of PCBs containing 54% chlorine was verified in a 12-year-old trout containing 26·2 ppm PCBs, the mass spectra of which were essentially identical with those of the isomers of Aroclor 1254. Zitko (*Bull. env. contam. & Toxicol.* (*U.S.*) 1974, **12**, 406) has demonstrated the uptake of Aroclor 1254 under experimen-

tal conditions in juvenile Atlantic salmon maintained in aerated fresh water containing, in a concentration of 1 g/litre, a suspension of silica contaminated with 0·1% Aroclor 1254. In fish killed after 24, 48 and 144 hours, whole-fish analyses indicated PCB concentrations of 19·9, 28·3 and 134·0 μg/g wet weight, respectively. Under comparable conditions, the uptake of chlorinated paraffins was much lower, concentrations equivalent to 0·2–0·75 μg/g (expressed as chlorine) being obtained after 48 or 144 hours. The GLC pattern of the absorbed PCB components compared with that of an Aroclor 1254 standard indicated a relatively high uptake of components with shorter retention times, particularly after the shortest exposure time. This preferential absorption was not observed, however, when food contaminated with Aroclor 1254 was fed to juvenile Atlantic salmon for 33–181 days, although again there was a marked discrepancy between uptake of the PCB and that of similarly administered chlorinated paraffins. When Aroclor 1254 was fed at a dietary level of 10 ppm, the PCB levels in the fish reached equilibrium, at 3·8–3·9 μg/g wet weight, within 33 days, but with a dietary level of 100 ppm residues of 13·9, 24·0 and 30·0 μg/g were found after 33, 109 and 181 days, respectively.

In a study of PCBs in the Lake Poinsett and Dry Lake waters in South Dakota, Greichus *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1973, **9,** 321) found that the concentration of organochlorine insecticides in bottom sediments was about seven times higher than that in the water, while fish in the two lakes contained concentrations of PCBs some 12 times higher than those in bottom sediments. There is thus a concentration gradient rising into the food chain. Greichus *et al.* (*loc. cit.*) found, moreover, that adult cormorants feeding on the fish in these two lakes contained concentrations of organochlorine compounds that were 250 times higher than those in the fish, while levels in the local pelicans were 280 times higher. PCBs in these two bird species were, respectively, 60 and 30 times higher than in the fish they fed on, and PCBs were detected in the cormorants' eggs. In both types of bird, the highest PCB concentrations were in the body fat (22·4 and 31·4 ppm wet weight on average in cormorants and pelicans, respectively) and paralleled the body-fat concentrations of total organochlorine compounds. Bogan & Bourne (*Nature, Lond.* 1972, **240,** 358) reported relatively low concentrations (0·1–1·0 ppm) of organochlorine residues in auks and shearwaters from the North Atlantic and higher levels (1–10 ppm) in more pelagic birds, such as kittiwakes, fulmars and storm-petrels. The highest concentrations, exceeding 10 ppm, occurred in large gulls and skuas feeding around trawlers in winter and seabird-breeding stations in summer. However, the finding that a fulmar carrying 42 ppm PCBs in its body fat had only 1·3 ppm in its stomach oil, which is fed to the chick, suggested only a low transfer from one generation to the next in food. Arctic glaucous gulls had, on average, 24 ppm PCBs in the liver, while one had 535 ppm in its body fat. One gull, observed to convulse, had a liver-PCB concentration of 311 ppm. The presence of PCBs in the Arctic, over 250 miles north of a major land mass, is somewhat disturbing.

PCBs in human foodstuffs

Ramsey (*Q. Bull. Ass. Fd Drug Off. U.S.* 1973, **37,** 43) has published an analysis of FDA findings during surveys of food intended for human consumption. No PCBs were found in fresh fruit and vegetables, and the most frequently contaminated food was fish, particularly freshwater fish. This was perhaps to be expected in view of the incorporation of PCBs into aquatic food chains noted above. Samples of shredded-wheat biscuits contained small concentrations of PCBs derived from paperboard dividers used in packaging. These dividers had been manufactured from recycled paper, which had included carbonless copy paper. In a survey started in 1971, about 19% of all the foods sampled contained PCBs. The overall average content of PCBs in packaged food was 0·1 ppm, and in some cases PCBs were found in the packaging materials but were not present in the food itself. Nearly 16% of the total samples of food examined contained PCB residues and were packaged in recycled materials, while contaminated food contained in virgin packaging material accounted for only 3·4% of the total samples.

Also in 1971, Shahied *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1973, **10,** 80) analysed 89 samples of paper pulp, newsprint, recycled paper and liner board and found PCB levels of more than 10 ppm in 11%, 6–10 ppm in 4% and 1–5 ppm in 25% of the samples. However, 58% of the total contained less than 0·5 ppm. The presence of PCBs in virgin newsprint and bond paper showed that recycling was not the only source and suggested that the manufacturing process might also be involved in PCB contamination. Early in 1972, Villeneuve *et al.* (*J. Ass. off. analyt. Chem.* 1973, **56,** 999) studied the PCB content of 569 packaging-material samples from 120 manufacturers in Canada, finding less than 1 ppm in 8·17%, 1–10 ppm in 14·9% and more than 10 ppm in 3·4%. Of 77 foods tested, only two contained more than 1 ppm PCBs. Of 73 foods tested for polychlorinated terphenyls (PCTs) in the same study, none contained more than 0·05 ppm. Levels of PCT residues in 445 packaging samples were reported to be less than 1 ppm in 84·5%, 1–10 ppm in 11·4% and more than 10 ppm in 4·1%.

That packaging may be carried out in such a way as to prevent any significant transfer of PCBs into the food is evident from a study by Stanovick *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1973, **10,** 101), who investigated the migration of Aroclor 1242 from treated paperboard containing this PCB at a level of 15 or 150 ppm. Food samples were stored at room temperature in jars lined with the paperboard for 2–90 days and were then assayed for PCBs. Barrier materials interposed between the paperboard liner and the food significantly reduced migration of the PCBs, the extent of the reduction being in inverse proportion to the gas-permeability of the barrier substance. Polyvinylidine chloride-coated paper prevented PCB migration for the full 90 days. Waxed glassine paper was the next most effective barrier, but polyethylene film was relatively ineffective over long periods of storage.

In his review of FDA food surveys, Ramsey (*loc. cit.*) stressed that, by 1973, PCB contamination of both food and packaging materials had shown some

reduction compared with the findings of the 1971 studies, and it seems that this trend is continuing. In addition to possible contamination from packaging, however, PCBs may also be introduced into the human diet in meat, milk and eggs derived from farm stock fed contaminated feedstuffs, and in this situation the concentration of residues demonstrated in natural food chains may again operate.

Teske *et al.* (*J. agric. Fd Chem.* 1974, **22**, 900) found that in chicks fed from 3 days of age on a diet containing 10 ppm Aroclor 1254, the PCB residues in dissected fat and muscle, respectively, were 110·6 and 109·0 ppm after 8 weeks and 49·5 and 102·8 ppm after 32 weeks. Depletion rates of administered Aroclor indicated a biological half-life of 2·93 weeks. Fat and muscle levels of residues in chicks fed 10 ppm in the diet for 52 days from the onset of egg production were 33·4 and 70·1 ppm, respectively. PCB residues in eggs from chicks fed this diet from 3 days of age reached a peak of 9·73 ppm about 4 weeks after the onset of egg production and subsequently decreased. When PCB treatment was delayed until the onset of egg-laying, the pattern was similar but the highest residue level was only 2·54 ppm. PCB concentrations in day-old chicks closely paralleled the concentrations in the eggs. Cooking has been shown to effect a significant reduction in the PCB residues in the edible tissues of PCB-treated chicks (Zabik, *Poult. Sci.* 1974, **53**, 1785).

Contamination of animal feeds may result from exposure during production, processing or storage, a few severe cases involving chicken feed having been due to the leaking of PCBs from a heat exchanger (Zabik, *loc. cit.*). The use of spent transformer oil in herbicide sprays and of plastics containing PCBs in silos and poultry-feed wrappers may also be a source of contamination. Savage *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1973, **10**, 97) took silage samples from 21 pit silos and ten upright silos (in which PCB sealants are sometimes used) on Colorado ranches. Detectable PCB contamination involved both pit and upright silos but was limited to samples from silos on only two ranches and in no case exceeded 0·08 ppm. One of these two ranches was concerned with dairy operations, but no PCBs were detected in milk from the silage-fed cattle.

It seems therefore that the problem of PCB contamination may be less severe than it seemed a few years ago. Nevertheless, a close watch must clearly be kept on the situation.

[P. Cooper—BIBRA]

β-AMINOPROPIONITRILE AND THE FOETUS

Rats and mice treated with β-aminopropionitrile (BAPN), the toxic principle of *Lathyrus odoratus*, have produced offspring with both horizontal and vertical forms of cleft palate but showing no retardation in growth (Posner, *Fd Cosmet. Toxicol.* 1972, **10**, 839). Further studies have been undertaken to determine the distribution of BAPN and its main metabolites in maternal and foetal tissues.

Metabolism and distribution

A study of the metabolism and teratogenicity of BAPN in rats has been published by Wilk *et al.* (*Teratology* 1972, **5**, 41). Females weighing 230–260 g were given an oral dose of 108–270 mg BAPN (as BAPN fumarate) on day 14, 15 or 16 of gestation, and were killed for examination on day 20, just prior to parturition. Others were given 500 mg of the major BAPN metabolite, cyanoacetic acid (CAA), or else 75 mg β-alanine, 75 mg cyanoethanol or 250 mg fumaric acid (as the sodium salt), again on day 14, 15 or 16. Embryos and placentas and maternal tissues, serum and urine contained both BAPN and CAA after BAPN administration. Peak concentrations of BAPN were reached in serum and tissues at 6 hours, but they declined after 9 hours and became negligible after 24 hours. In these rats, tissue and serum concentrations of CAA, unlike those of BAPN, showed a gradual increase and remained high at 24 hours, by which time over 60% of a dose of 270 mg BAPN had been excreted unchanged in the urine and about 3% had been excreted as CAA.

The incidence of cleft palate in the embryos correlated with the dose of BAPN given and with the observed concentrations of BAPN in maternal and embryonic tissue, indicating that BAPN itself was the teratogen. This was supported by the finding that despite the appearance of high concentrations of CAA in maternal and embryonic tissues, direct administration of CAA failed to produce cleft palate. No abnormalities followed dosing with β-alanine or cyanoethanol, two probable metabolites of BAPN, or with sodium fumarate. Although similar tissue concentrations of BAPN were produced by doses given on any of the 3 days, a positive correlation of cleft-palate incidence with dosage and tissue levels appeared only with the dose given on day 15, when embryonic concentrations of 106, 42 and 16 μg BAPN/g tissue were associated with a cleft-palate incidence of 98, 28 and 0% respectively.

Waddell *et al.* (*ibid* 1974, **9**, 211) injected BAPN labelled with ^{14}C iv into mice on day 12·5 or 15·5 of gestation. After 20 minutes, whole-body autoradiography showed the highest radioactivity to be in the salivary gland and in the urine present in the bladder. Some activity was also demonstrated in the pyloric but not the cardiac stomach. The radioactivity in foetal tissues was in general less than that in the maternal tissues, and was most intense throughout the central nervous system. After 1 hour, the foetal radioactivity exceeded that in maternal tissues, with highest concentrations in the central nervous tissue, eye, yolk sac and the tissues lining the oral cavity. After 3 and 9 hours, maternal concentrations of radioactivity were high in the eye, liver, kidney, salivary gland, intestinal wall, inner ear, aorta and vertebral ligaments. When labelled BAPN was injected sc in conjunction with a large excess of unlabelled compound, intense radioactivity appeared in the salivary gland, bone,

liver, kidney, intestines and stomach contents. Chromatographic studies on homogenates of representative sections of these sc-treated mice showed that 20 minutes after the injection about 66% of the radioactivity was in the form of unchanged BAPN and some 33% was CAA; 1 and 3 hours after treatment about half of the dose had been metabolized, and at 9 and 24 hours about 66% of the radioactivity was in the form of CAA.

BAPN in the chick embryo

The effect of BAPN on the developing chick embryo has been studied by Hall (*ibid* 1972, **5**, 81), who injected a dose of 1·25, 0·625, 0·312 or 0·156 mg BAPN into the chorioallantoic membrane of 7-day eggs of *Gallus domesticus*, which were examined after incubation for 10, 12 or 14 days. No embryo survived to 14 days after injection of either of the two higher doses, and the survival rate after the two lower doses was about 50%. The mean body weight of the embryos was lower than that of controls of the same age, but the difference was statistically significant only in embryos incubated for 14 days. Retardation of growth was also indicated by a significant reduction in third-toe length at 14 days. The tibia and membranous bones of the lower jaw and upper beak showed gross defects, and bending of the mandible, tibia and fibula and shortening of the upper jaw occurred. Stimulation of osteogenesis on the concave surface of the bent tibia led to the appearance of an exostosis. Acid mucopolysaccharides of the affected tissues showed abnormal staining properties, and the histological and cytological changes observed were primarily associated with proliferation of the fibrous and cellular layers of the tibial periosteum.

BAPN in the baboon

Steffek & Hendrickx (*ibid* 1972, **5**, 171) found that foetal resorption occurred by day 63 in a pregnant baboon given 200 mg BAPN/kg/day im on days 38–50 of gestation, and by day 54 in another given 300 mg BAPN/kg/day orally on days 37–48. A third baboon given 500 mg BAPN/kg/day iv on days 43–48 produced a macerated foetus with spina bifida, delivered by Caesarian section after threatened abortion on day 74 of gestation. In a comparative study on another lathyrogen, aminoacetonitrile (AAN), two abortions and one foetal resorption occurred in three baboons given 20, 60 and 70 mg AAN/kg/day on days 38–50, 38–48 and 38–41, respectively. The middle dose level was given orally and the other two im. AAN given orally in doses of 75 mg/kg/day on days 43–48 produced a foetus with digital defects in one baboon and a set of twins with abnormally curved limb bones and cleft palate in another. In other baboons, however, treatment with 20 or 40 mg AAN/kg/day im on days 38–50, or with 60 or 130 mg AAN/kg/day orally on days 40–48, 45–48 or 46–48 did not prevent the delivery of normal offspring.

[P. Cooper—BIBRA]

Fd Cosmet. Toxicol. Vol. 13, pp. 581–594. Pergamon Press 1975. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

2899. The protective effects of plant fibre

Ershoff, B. H. & Thurston, E. W. (1974). Effects of diet on amaranth (FD&C Red No. 2) toxicity in the rat. *J. Nutr.* **104,** 937.

Amaranth has recently been cleared of suspicions of teratogenicity (*Cited in F.C.T.* 1975, **13,** 473) but, because of a paucity of metabolic data in primates, a US *ad hoc* advisory committee has recommended that intake should be restricted to 2 mg/kg/day (*Food Chemical News* 1975, **16** (43), 40). The toxicity of amaranth, unlike that of butter yellow, was not increased by protein deficiency (Albrecht *et al. Fd Cosmet. Toxicol.* 1973, **11,** 383), but the marked ability of another dietary component to affect its toxicity is demonstrated in the present study.

Rats fed amaranth for 21 days at a level of 5% in a stock ration based on common feed ingredients differed from the controls little, if at all, in respect of weight gain, gross appearance or survival. However, the same level of amaranth fed in a purified, vitamin-supplemented diet containing 66% sucrose, 24% casein, 5% salt mixture and 5% cottonseed oil produced a virtual cessation of growth, an unthrifty appearance and death within 2 wk. At a level of 2·5% in the purified diet, the effects of amaranth were less severe, being limited to a retardation of weight gain in males. Supplementation of the purified 5%-amaranth diet with increased amounts of the vitamins or nutrients or with lemon bioflavonoid complex, desiccated whole liver or zinc sulphate had little or no protective effect. On the other hand, a moderate degree of protection was afforded by 10% dried yeast or pectin, or by 20% sesame-seed meal or whole-wheat flour, and complete protection resulted from the inclusion of 5 or 10% cellulose or 10% alfalfa meal, watercress powder or parsley powder. Alfalfa-pulp residue (10%) was equally potent, but dried alfalfa juice and ash at levels equivalent to those in 10% alfalfa meal were ineffective. This suggested that the fibre content (about 20%) of the plant materials was the protective agent, but since 2% cellulose was found to afford little protection, it appeared that hemicelluloses and/or other plant ingredients played the major protective role.

2900. Effect of bile salts on tartrazine metabolism

Allan, R. J. & Roxon, J. J. (1974). Metabolism by intestinal bacteria: The effect of bile salts on tartrazine azo reduction. *Xenobiotica* **4,** 637.

Tartrazine given orally undergoes reductive cleavage to yield sulphanilic acid as a result of bacterial activity in the intestine (*Cited in F.C.T.* 1969, **7,** 87; Ryan *et al. Fd Cosmet. Toxicol.* 1969, **7,** 287 & 297). Even after parenteral administration, considerable amounts of tartrazine are excreted in the bile, and are thus still exposed to azo reduction by the gut flora (*Cited in F.C.T.* 1973, **11,** 679). Basic requirements for azo reduction *in vitro* by the bacterium *Proteus vulgaris* have been elaborated (Roxon *et al. Fd Cosmet. Toxicol.* 1966, **4,** 419) and the rate of reduction has been shown to be maximal in suspensions starved for 20–30 hr (*idem, ibid* 1967, **5,** 645). This was the rate that best accounted for the *in vivo* finding that in the rat and man, respectively, 60 and 90% of an oral dose of tartrazine was excreted as sulphanilic acid in the urine within 48 hr (Jones *et al. Fd Cosmet. Toxicol.* 1964, **2,** 447). It was thought that starvation of the washed suspensions made the cells more permeable and it seemed possible that other factors might increase the rate of bacterial azo reduction *in vivo*. With this in mind, the possible role of bile salts has now been investigated.

Bile salts incubated with tartrazine in washed cell suspensions of *P. vulgaris* were found to have a negligible effect on the rate of reduction of the colouring when the cells were harvested from cultures that had reached the stationary, post-multiplication phase. However, in suspensions of cells taken from cultures still in the actively-growing phase, the rate of tartrazine reduction was enhanced by bile salts in a dose-dependent manner, a 1% concentration of bile salts producing a fivefold increase in activity. This enhancement occurred with a negligible time lag. Cells from the two different phases incubated in the absence of bile salts showed low and comparable activity. The mechanism of action of bile salts was not elucidated, although it seemed probable that they might modify cell permeability to tartrazine or co-factors.

In view of these findings, it is suggested that a study of the effects of bile salts should be included as a routine measure in any *in vitro* studies of metabolism by the gut flora.

FLAVOURINGS, SOLVENTS AND SWEETENERS

2901. Alcohol imperils the genes

Badr, F. M. & Badr, Ragaa S. (1975). Induction of dominant lethal mutation in male mice by ethyl alcohol. *Nature, Lond.* **253,** 134.

The toxicity of ethanol is characterized by a wide range of adverse effects, both acute and chronic (*Cited in F.C.T.* 1970, **8,** 433). Studies of the effect of ethanol on reproductive performance go back many years, but

it has been assumed that the alcohol poses no genetic hazard. That this view may not be wholly justified is indicated by the study cited above, which shows that under certain conditions ethanol can act as a mutagen.

Male mice were given 0·1 ml of 40 or 60% ethanol by gastric intubation on three consecutive days and were then mated with untreated females. The litter sizes following matings on days 14–17 after ethanol treatment differed significantly from those in the control (water-treated) group, the mean value being 4·4 compared with a control mean of 7·8. A two- to four-fold increase in the number of dead implantations was found in females mated 4–13 days after ethanol treatment of the males, and this was associated with a proportional decrease in the number of live embryos. The calculated dominant lethal mutation index was increased for matings taking place between days 1 and 13 in the group treated with 40% ethanol, with a peak for matings on days 9–13. In the 60%-ethanol group, the index increased more rapidly and abruptly, with frequencies at days 4–8 and 9–13 of 57·4 and 67·3, respectively, compared with 30·6 and 46·3 for the 40%-ethanol group at the same mating intervals.

These figures indicate a positive dose–response relationship between ethanol and dominant lethal mutation. It also appears that ethanol induces dominant lethal mutations both in the epididymal spermatozoan and late spermatid stage of spermatogenesis, the induction being more pronounced in the latter stage. Further investigations will be required to determine whether ethanol might produce a wider range of genetic effects than dominant lethal mutation.

2902. More about propylene glycol in animal experimentation

Dean, Margaret E. & Stock, B. H. (1974). Propylene glycol as a drug solvent in the study of hepatic microsomal enzyme metabolism in the rat. *Toxic. appl. Pharmac.* **28**, 44.

Although the pharmacological effects of propylene glycol (1,2-propanediol; PG) are slight, warnings have been given that the possible interference of this solvent in the results of pharmacological testing should not be completely discounted (*Cited in F.C.T.* 1972, **10**, 715). The authors cited above have reported significant effects from PG used as a solvent for compounds that are metabolized by the hepatic microsomal enzymes.

Microsomal preparations from the livers of rats treated ip with 4 ml PG/kg twice daily for 3 days showed a significant increase in their ability to metabolize aniline and *p*-nitroanisole *in vitro*. Aminopyrine demethylation, however, was significantly reduced. The metabolism of *p*-nitrobenzoic acid was not affected. When given at a dose level of 1, 2 or 6 ml/kg, PG had less effect on the metabolism of aniline and *p*-nitroanisole than at 4 ml/kg. Aminopyrine demethylase, however, showed a progressive decrease with increasing dose, while *p*-nitrobenzoic acid metabolism showed a biphasic response, with values somewhat below the control of 2 ml/kg and slightly raised at 6 ml/kg. PG treatment of the rats did not change the microsomal concentration of cytochrome *P*-450. When PG was added *in vitro* to isolated liver-microsomal preparations, it failed to induce the changes seen after animal dosing.

Hexobarbitone sleeping time and zoxazolamine paralysis time were increased in rats treated with PG. When phenobarbitone (75 mg/kg) was given concurrently with 4 ml PG/kg on three successive days, the increase in metabolism of aniline and *p*-nitroanisole was found to be additive when compared with that produced by either drug alone, but the depression of aminopyrine demethylation associated with PG alone was reversed by concurrent administration of phenobarbitone.

Thus, despite the apparent suitability of PG for use as a solvent in animal experiments, it cannot be regarded as completely inert in the rat. Its effect on liver metabolism resembles that of dimethyl sulphoxide, and may be attributable to some physical distortion produced in the endoplasmic reticulum of the liver.

ANTIOXIDANTS

2903. Behavioural effects of BHA and BHT

Stokes, J. D & Scudder, C. L. (1974). The effect of butylated hydroxyanisole and butylated hydroxytoluene on behavioral development of mice. *Dev. Psychobiol.* **7**, 343.

The principal effect of both butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in experimental animals is a stimulation of the microsomal enzymes of the liver, with an associated enlargement of that organ (Allen & Engblom, *Fd Cosmet. Toxicol.* 1972, **10**, 769). However, neither this nor other adverse effect has so far been detected in rats and mice maintained on dietary levels of 0·5% BHA or BHT, and on this basis the Joint FAO/WHO Expert Committee on Food Additives has estimated the total acceptable daily intake of BHA and/or BHT for man to be up to 0·5 mg/kg.

In the study cited above, a dietary level of 0·5% BHA or BHT was fed for 3 wk to weanling mice, whose parents had been maintained on the same level of BHA or BHT during the entire mating, gestation and pre-weaning period. Assessments were then made of social behaviour, learning ability (conditioned avoidance of electric shocks in a multi-chambered climbing apparatus), aggression after a 3-wk period of isolation, orientation reflexes and psychomotor activity. BHA treatment was associated with a significant increase in exploration activity, a decrease in sleeping and self-grooming, slower learning and a decrease in the orientation reflex. BHT also decreased sleeping, increased social and isolation-induced

aggression, and prevented learning altogether under the experimental conditions employed. Neither compound, however, affected digging, stereotypic behaviour, freezing, carrying, ingestion, being groomed, grooming others, or contactual or sexual behaviour. Aggression was unmodified in 9-wk-old mice, previously untreated but fed BHA or BHT for the third week of a 3-wk period of isolation, suggesting that treatment was effective only at a very early stage of development.

Climbing times in the learning study were unchanged by either BHA or BHT, ruling out the possibility of a neuromuscular deficit, a soporific effect or a weakening of reflex activity, and the animals were not ataxic or impaired in motor co-ordination. A pilot study has revealed a decrease in serotonin and cholinesterase activity and changed noradrenaline levels in the brains of newborn mice exposed *in utero* to BHA or BHT fed to the pregnant mice at dietary levels of 0·5% (Stokes *et al. Fedn Proc. Fedn Am. Socs exp. Biol.* 1972, **31**, 596), and it is suggested that these changes may play a role in the behavioural modifications now reported.

[It has been suggested that ingestion of food additives may be associated with hyperkinesis and learning difficulties in children, and some studies in this field have recently been undertaken in the USA (*Food Chemical News* 1974, **16** (13), 2; *ibid* 1974, **16** (39), 30). The present study may pave the way for an examination of the behavioural effects of other food additives.]

2904. The fate of thiodipropionates

Reynolds, R. C., Astill, B. D. & Fassett, D. W. (1974). The fate of [^{14}C]thiodipropionates in rats. *Toxic. appl. Pharmac.* **28**, 133.

Thiodipropionates have been used in foods and food packaging as antioxidants and stabilizers. The fate of thiodipropionic acid (TDPA), of didodecyl thiodipropionate (DDTDP) and of a polyester of TDPA with cyclohexane-1,4-dimethanol partially terminated with stearyl alcohol (POLY-TDPS-2000; TDPS; mol wt 1800–2200) has been studied after oral administration to rats, each compound being labelled with ^{14}C in the carboxyl group.

The radioactivity from single intubated doses of 550–650 mg [^{14}C]TDPA/kg was almost entirely absorbed from the gut and most was excreted within 4 days, 78–88% being recovered in the urine, 0·1–0·9% in the faeces and 3–8% as respiratory CO_2 in this period. Over 90% of the total eliminated was accounted for in the first 24 hr. Radioactivity in the tissues was increased to less than 1·5 times the background level, and had returned to normal 34 days after treatment. Urinary activity after these doses was due almost entirely to unchanged TDPA, whereas with a dose of 3 mg/kg an acid-labile conjugate, apparently not a glucuronide, appeared.

Within 4 days of administration of a single dose of 107 or 208 mg DDTDP/kg, 85–88% of the ^{14}C activity appeared in the urine, either as the free compound or as an acid-labile conjugate, with 1·8–3·5% in the faeces and 3–4% in the expired air. Apart from body fat, which showed a rise in activity at 4, 8 and 32 days, no tissues showed any significant increase in activity.

Comparable levels of excretion by all three routes followed administration of [^{14}C]TDPA (241 mg/kg body weight) or [^{14}C]DDTDP (166 mg/kg) in the feed over a 5-hr period rather than in a single intubated dose. When 4·7–5·6 mg [^{14}C]TDPS/kg was given similarly in the feed for 5 hr, 95% was eliminated in the urine, 0·7% in the faeces and about 6% in the expired air within 4 days. Tissue radioactivity was slightly above the background level at 4 days but had become normal at 34 days. About 65% of the urinary radioactivity was in the form of TDPA or a conjugate.

AGRICULTURAL CHEMICALS

2905. Getting rid of aldrin

Rumsey, T. S. & Bond, J. (1974). Effect of urea, diethylstilbestrol, and type of diet on the distribution of aldrin and dieldrin residues in finished beef heifers. *J. agric. Fd Chem.* **22**, 664.

The storage of aldrin and dieldrin in body tissues is known to be affected by the level of dietary protein and by other substances that influence metabolism and enzyme activity. The effect of different nutritional regimens and of diethylstilboestrol (DES) implants on aldrin and dieldrin residues derived from the environment has therefore been studied in beef cattle.

Calves were reared on a corn-based starter diet (and subsequently on a forage diet) containing soyabean meal or urea as a supplementary nitrogen source. Half of the animals in each group received, in addition, implants of 12 mg DES on days 168 and 346. The animals were given 1 mg aldrin/kg/day in the feed from day 42 of the experiment, and tissue samples taken when the animals were killed, about 16 months later, were used for the estimation of aldrin and its metabolite, dieldrin. The average concentrations of aldrin and dieldrin, respectively, in fatty tissue were 7 and 14 times those in muscle tissue and organs, and the average tissue concentration of dieldrin was more than 100 times that of aldrin in any one tissue. The different dietary regimens and the DES implants made no difference to the tissue distribution of residues of the pesticides, but implanted animals showed a lower average concentration of aldrin and dieldrin in all fatty tissues while urea-fed animals showed a higher one than was found in controls. This difference may reflect the increase in metabolic rate that apparently occurs in DES-treated cattle, and a reduction in fat cover in those fed urea.

2906. A metabolic mystery of DDT

Gingell, R. & Wallcave, L. (1974). Species differences in the acute toxicity and tissue distribution of DDT in mice and hamsters. *Toxic. appl. Pharmac.* **28**, 385.

DDT appears to exert a neurotoxic effect by interfering with metabolism in the brain. This aspect of DDT activity has received further attention in a study designed to provide some explanation for the marked difference between the acute neurotoxicity of DDT in mice and in hamsters. The acute LD_{50} of *p,p'*-DDT in the Swiss mouse was 305 mg/kg in males and 318 mg/kg in females, but no Syrian hamsters died within 24 hr of receiving a single oral dose of 1600 mg/kg, and only three hamsters died out of a group of five given five successive doses each of 1000 mg/kg within a total period of 48 hr. The primary metabolites of DDT (DDD and DDE) given in doses up to 1600 mg/kg caused no deaths or neurotoxic signs in mice, indicating that DDT itself was the neurotoxic agent.

Brain concentrations measured 12 hr after administration of an oral dose of 500 mg DDT/kg were about twice as high in mice as in hamsters, but were similar in mice dying after a dose of 500 mg/kg and in hamsters killed by five doses of 1000 mg/kg. It thus appears that the brain of each species is equally sensitive to DDT and that the difference possibly lies in the permeability of the blood–brain barrier. When the animals were maintained on a diet containing 250 ppm DDT for 6 wk, total DDT, DDD and DDE residues in fat and liver were 7–8 times higher in mice than in hamsters, a difference only partly accounted for by the higher rate of food consumption achieved by the mouse (220 g/kg/day compared with about 70 g/kg/day for the hamsters). In the mouse, the liver contained relatively high concentrations of DDE, while in the hamster liver, DDD predominated. Little species difference was apparent in the faecal and urinary excretion of radioactivity after a single oral dose of 25 mg [^{14}C]DDT/kg. However, when this excretion study was repeated after the feeding of a diet containing 250 ppm DDT for 6 wk, a roughly threefold increase in urinary ^{14}C excretion occurred in the hamster but not in the mouse. Moreover, similar pretreatment with dietary DDT markedly decreased hexobarbitone sleeping time in hamsters but had less effect in mice, while phenobarbitone pretreatment significantly decreased sleeping time in both species. There was no histological evidence of liver damage in the DDT-pretreated mice to account for the apparent inability of DDT to induce its own metabolism in that species as it appears to do in hamsters, but the increased capacity for metabolism in the hamsters undergoing prolonged treatment with DDT could account for the higher tissue residues found in mice.

2907. Dichlobenil and the skin

Deeken, J. H. (1974). Chloracne induced by 2,6-dichlorobenzonitrile. *Archs Derm.* **109**, 245.

The herbicide dichlobenil (2,6-dichlorobenzonitrile; Casoron) is degraded microbiologically in the soil to 2,6-dichlorobenzamide, which is also found in plants treated with the substance (*Cited in F.C.T.* 1973, **11**, 1111).

The paper cited above reports six cases of chloracne encountered among chemical workers handling dichlobenil in bulk. Affected workers were either engaged in bagging the herbicide or in dumping the powder into a glue solution, in which it is mixed ready for spraying on to an inert clay carrier. The time elapsing between initial exposure to dichlobenil and the appearance of an acneiform eruption was up to 5 months. The location and type of eruption in these workers were comparable to those reported in other cases of chloracne, the skin areas usually affected being the forehead, cheek, jaw and suborbital regions. Standard forms of treatment were all disappointing until the patients had been withdrawn from further exposure to dichlobenil, when the condition rapidly resolved. It is suggested that persons with darker complexions and a history of teenage acne may be more liable than others to develop dichlobenil acne.

2908. Excreting ethylene thiourea ...

Newsome, W. H. (1974). The excretion of ethylenethiourea by rat and guinea pig. *Bull. env. contam. & Toxicol.* (*U.S.*) **11**, 174.

Ethylene thiourea (ETU), which has been identified as a contaminant formed in ethylenebisdithiocarbamate fungicides during storage, has been reported to produce goitre and thyroid hyperplasia in rats (*Cited in F.C.T.* 1973, **11**, 702) and foetotoxicity and congenital malformations in rats and to a less marked degree in rabbits (*ibid* 1974, **12**, 282).

Newsome (cited above) has determined the rate of excretion of ingested ETU by rats and guinea-pigs. Male rats and male guinea-pigs were given a single intragastric dose of 20 mg ETU/kg. Urinary excretion in both species was rapid, with rats excreting more than half of the dose unchanged in the urine within 24 hr and guinea-pigs only slightly less. The total recovery of ETU in guinea-pigs was 48·2% and in rats 66·4%, faecal elimination within 48 hr being only 0·78 and 1·06% respectively. This difference in total recovery was possibly attributable to interspecies variation in metabolism. There was a striking concentration of ETU in the thyroid in both species, levels of 0·824 ppm in this organ in rats and 0·751 ppm in guinea-pigs contrasting with mean concentrations of the order of 0·01–0·09 ppm in liver, kidney, heart and skeletal muscle. It is possible, since the analytical method used measured only the parent compound, that metabolic residues so far undetected may also occur in the tissues.

2909. ... and its fate in the cooking pot

Watts, R. R., Storherr, R. W. & Onley, J. H. (1974). Effects of cooking on ethylenebisdithiocarbamate degradation to ethylene thiourea. *Bull. env. contam. & Toxicol.* (*U.S.*) **12**, 224.

The cooking of foods containing residues of fungicides based on ethylenebis(dithiocarbamate) (EBDC) has been shown to result in the degradation of the fungicide to ethylene thiourea (ETU) (*Cited in F.C.T.* 1974, **12**, 779). The latter compound has already been reported to be teratogenic (*ibid* 1974, **12**, 282) and to have carcinogenic and other toxic effects on the thyroid (*ibid* 1973, **11**, 702). Since EBDC residues are present in a variety of foods, it is important to determine the possible significance of the quantities of ETU that might result from cooking such foods.

Chopped spinach, carrots and potatoes were mixed with 10 ppm EBDC fungicide shortly before or immediately after cooking, and the samples were then analysed in these pairs so that any differences in ETU content could be directly attributed to the effects of cooking. The fungicides used in the experiment were maneb (manganous EBDC), Dithane M-45 and Manzate (zinc manganous EBDCs) and Polyram (ammoniated zinc EBDC with free ethylenebis(dithiocarbamic acid). After normal cooking procedures the quantities of ETU formed in the vegetable and cooking-water varied from 11·2% (w/w) of the maneb added to potatoes to 26·5% of the Polyram added to spinach. Thus significant amounts of ETU were formed from all the EBDCs tested in each type of vegetable, and similar quantities of ETU resulted from boiling the fungicides with water alone. ETU itself is stable in boiling water, as was evident from the finding that in a pair of spinach samples fortified with 10 ppm ETU before or after boiling the recoveries were 87 and 85%, respectively.

In assessing the importance of residues in foodstuffs, it is clear that the EBDC residue must be taken into account together with any initial ETU residue.

2910. Mirex in the environment

Ivie, G. W., Gibson, J. R., Bryant, H. E., Begin, J. J., Barnett, J. R. & Dorough, H. W. (1974). Accumulation, distribution, and excretion of mirex-^{14}C in animals exposed for long periods to the insecticide in the diet. *J. agric. Fd Chem.* **22,** 646.

Mirex is dodecachloropentacyclo-[5,3,0,$0^{2,6}$,$0^{3,9}$, $0^{4,8}$]decane and is used primarily to control the fire ant (Solenopsis spp.). It is suitable and highly lipophilic and may therefore be expected to have a wide environmental impact. Mirex has been shown to induce liver damage and cataract in rats (*Cited in F.C.T.* 1971, **9,** 590).

The paper cited above describes the feeding of rats with [^{14}C]mirex at 0·3 ppm, or with [^{14}C]mirex diluted tenfold with unlabelled compound at 3 or 30 ppm for 6 or 12 months, the feeding of the same dietary levels of labelled mirex to Japanese quail (*Coturnix coturnix japonica*) for up to 16 months, and the feeding of a diet containing 50 ppm [^{14}C]mirex to mosquito fish (*Gambusia affinis*) for up to 56 days. All three species acquired high body burdens of mirex, and no metabolism was detected. Maximal residues were found in adipose tissue, rising after 16 months to concentrations equivalent, in rats and male quail respectively, to some 120 and 185 times the dietary concentration, irrespective of the dietary level fed. No plateau level was detected in any of the three species. Female quail showed lower tissue residues of mirex than males, considerable quantities being eliminated from laying birds in the egg yolk. No adverse effects on hatchability or the growth and survival of chicks were apparent, however, even with egg-yolk concentrations of up to 200 ppm mirex.

In female rats, residues in body fat declined by some 40% after transfer of the animals to a mirex-free diet for 10 months. In fish, the half-life of mirex was about 130 days, and in the fat of male and female quail it was 30 and 20 days, respectively. Rats excreted polar photoproducts of mirex relatively rapidly after their administration by oral intubation for 7 days at a dose level of 0·1 mg mirex equivalents/kg/day. This indicates that the potential hazard of mirex accumulation might be lessened by environmental photodecomposition.

2911. Filling in on pentachlorophenol

Ahlborg, U. G., Lindgren, J.-E. & Mercier, M. (1974). Metabolism of penetachlorophenol. *Arch. Tox.* **32,** 271.

Schwetz, B. A., Keeler, P. A. & Gehring, P. J. (1974). The effect of purified and commerical grade pentachlorophenol on rat embryonal and fetal development. *Toxic. appl. Pharmac.* **28,** 151.

Pentachlorophenol (PCP) and its water-soluble derivatives have been used as fungicides and bactericides in a wide range of products, and the need to prevent their absorption through the skin is well recognized. The metabolism of PCP in rats has been reported (*Cited in F.C.T.* 1973, **11,** 1152), and the first paper cited above gives the results of further metabolic studies in which rats and mice were given ^{14}C-labelled PCP in an oral or ip dose of 10–25 mg/kg. After injection, most radioactivity was recovered from the urine within 40 hr (some 70% within 24 hr), with 41–43% of the activity representing unchanged PCP. After oral doses, urinary excretion of radioactivity was lower than that after injection, and mice showed a 50% lower recovery in the urine than did rats. Unconjugated tetrachlorohydroquinone was a major metabolite, representing 24% of the activity excreted by the mouse (but only 5% of that excreted by the rat) within 24 hr. A second important metabolite was probably a mixture of conjugates of PCP and this hydroquinone. Acid hydrolysis converted all the radioactive components of the urine to PCP and tetrachlorohydroquinone, the latter accounting for 43 and 46% in rats and mice respectively. Analysis of the urine of two spraymen who had been occupationally exposed to PCP showed that tetrachlorohydroquinone also occurs in human urine after PCP exposure.

The paper by Schwetz *et al.*, cited above, describes the oral administration of purified and commercial grades of PCP to rats on days 6–15, 8–11 or 12–15 of gestation at levels up to the maximum tolerated dose of 50 mg/kg/day. The incidence of resorptions and of subcutaneous oedema, ureteric dilatation and abnormalities of the skull, vertebrae and ribs among the offspring increased with increasing dosage over the range 15–50 mg/kg/day with commercial-grade PCP and 30–50 mg/kg/day with purified PCP. The no-effect level for the commercial grade was 5 mg/kg/day administered on days 6–15 of gestation. Purified PCP, with a low non-phenolic content, was slightly more toxic than the commercial grade, judged by its effect on maternal weight gain, the rate of foetal resorption, foetal body measurements and the incidence of foetal abnormalities. It was estimated that the dose of PCP that would be lethal to 50% of the embryos *in utero* was 16 mg/kg/day for the pure material and 44 mg/kg/day for the commercial product. Embryos appeared to be more susceptible to the effects of PCP during gestation days 8–11 than during

days 12–15. Exposure during days 8–11 produced effects similar to those of exposure during days 6–15.

2912. More toxic effects of tetrachlorodibenzo-*p*-dioxin

Vos, J. G., Moore, J. A. & Zinkl, J. G. (1974). Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57Bl/6 mice. *Toxic. appl. Pharmac.* **29,** 229.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin ('dioxin'; TCDD) is ranked as one of the most toxic compounds known. Its chief significance is as an impurity in 2,4,5-trichlorophenol produced by alkaline hydrolysis of 1,2,4,5-tetrachlorobenzene and in 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). In addition to causing chloracne, TCDD present as a low-level contaminant (0·05–0·5 ppm) in 2,4,5-T, has been implicated in the teratogenicity caused by this herbicide in mice, but the position in this respect is still not altogether clear (*Cited in F.C.T.* 1972, **10,** 722). Further toxic effects of TCDD are described in the paper under review.

Mice were given single doses of 100–200 μg TCDD/kg or weekly doses of 0·2–25 μg/kg, all the doses being given orally in corn oil. Half of the mice in the weekly-treated groups were killed after 2 wk and the survivors after 6 wk. The acute oral LD_{50} was 114 μg/kg, and mice dying after such a dose consistently showed depletion of thymus and spleen, with centrilobular liver lesions. In addition, they showed gastrointestinal oedema and haemorrhage, with intra-orbital blood effusion, which often caused protrusion of the eye. The subacute toxic effects of TCDD included death between days 35 and 40 in three of the 17 animals given 25 μg/kg; liver weight was increased and thymus weight was reduced both in this group and in those given 1·0 and 5·0 μg TCDD/kg. The total neutrophil count rose significantly in mice given six doses of 25 μg/kg. In the same animals, the haemoglobin and mean corpuscular haemoglobin values were reduced, while total serum proteins and α-, β- and γ-globulins fell significantly. Some effects on serum proteins were also evident at the lower dosage levels. Porphyria appeared and was probably associated with the liver damage, which included centrilobular degeneration and necrosis accompanied by cellular infiltration and ceroid pigmentation. Progressive hepatic lipid accumulation occurred, ranging from localized centrilobular deposits with doses of 0·2 μg/kg to deposits affecting all hepatocytes with doses of 25 μg/kg.

[Marked liver hypertrophy and thymic regression were seen also in rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a study reported by Allen *et al.* (*Fd Cosmet. Toxicol.* 1975, **13,** 501).]

2913. TOK strikes the foetal lung

Kimbrough, R. D., Gaines, T. B. & Linder, R. E. (1974). 2,4-Dichlorophenyl-*p*-nitrophenyl ether (TOK). Effects on the lung maturation of rat fetus. *Archs envir. Hlth* **28,** 316.

2,4-Dichlorophenyl-*p*-nitrophenyl ether (TOK) is a selective herbicide and has been reported to increase mortality among the offspring of animals fed 100 ppm or more in the diet (*Cited in F.C.T.* 1972, **10,** 428).

In the study cited above, technical (89%) TOK was fed at dietary levels of 20, 100 or 500 ppm to rats in a two-generation experiment, and technical and pure (99+%) samples were given to female rats by oral intubation on specified days of gestation only. Technical TOK fed at the 20 ppm level did not affect the survival of offspring, but at 100 ppm survival to weaning was significantly reduced, and at 500 ppm death during the neonatal period was almost invariable. Food consumption and weight gain were not significantly affected in any group.

Treatment of females with 50 mg TOK/kg/day on days 7–15 of gestation reduced the number of live births per litter from 12·7 (in controls) to 2·2 and 2·7 (for pure and technical grades, respectively) and none of the pups survived to weaning. A less marked reduction in survival (to approximately 50% of the control figure) followed treatment with 20 mg/kg/day over the same period. Technical TOK given in daily doses of 10 mg/kg had no adverse effect and a similar absence of effect was noted following treatment on days 7–15 with 2,7-dichlorodibenzodioxin, a probable though not chemically confirmed contaminant of technical TOK. The dioxin was given in daily doses of 0·04 mg/kg, equivalent to a contamination level of 2000 ppm. Rats fed 500 ppm TOK and examined by Caesarean section on day 20 of gestation showed an average of 11·8 live pups per litter, compared with 12·4 in controls, but the TOK-exposed pups were cyanosed and died within 1–3 hr. Rats intubated with 50 ppm technical TOK on days 7–18 had live pups delivered by Caesarean section on day 21, but these too were cyanosed, a condition that intensified during the subsequent 45–60 min and usually ended in death. Foetal lungs from exposed groups were characterized by poor expansion and the presence of cuboidal alveolar epithelium. Examination of foetal lungs by electron microscopy revealed evidence of cellular degeneration.

FEED ADDITIVES

2914. Photosensitization to quindoxin

Scott, K. W. & Dawson, T. A. J. (1974). Photo-contact dermatitis arising from the presence of quindoxin in animal feeding stuffs. *Br. J. Derm.* **90,** 543.

Quindoxin (quinoxaline 1,4-di-*N*-oxide) has been used as a growth-promoting additive in animal feeds at levels of 20–50 mg/kg feed. It is now reported to have been withdrawn from the market on account of indications of carcinogenicity in experimental animals given high oral doses.

Prior to its withdrawal, however, seven men and ten women employed in pig farming or the delivery of animal feeding stuffs developed a rash, with lesions more marked in areas exposed to light. The rash resembled that of a dry eczematous dermatitis with

a fissuring tendency, like that caused by cement. Photopatch and closed-patch tests were carried out using quindoxin, ethoxyquin and copper sulphate. the two latter compounds being widely used animal-feed additives. All 17 patients gave strong positive reactions at 48 hr with 0·1% quindoxin in yellow petrolatum in the photopatch test. Seven gave a positive and ten a negative result with quindoxin in the closed-patch test at 48 hr, but eight of the non-reactors were examined again at 72 hr, when each gave a positive result. Negative reactions to ethoxyquin and copper sulphate were obtained throughout. A battery of standard closed-patch tests showed eight positive results among seven of the quindoxin-sensitive patients; six gave a single reaction to balsam of Peru, benzocaine. chlorquinaldol, epoxy resin or wood tar, and one reacted to both chinoform and chlorquinaldol.

When five patients, who had already given positive reactions in photopatch testing, were tested with 0·25% quindoxin in yellow petrolatum applied under a black light-proof textile, only two gave a faintly positive reaction at 72 hr, although all five reacted positively within 24 hr once the light-protective cover was removed. Thus, while quindoxin is a potent photosensitizer, it appears to be only a weak sensitizer in the absence of light.

PROCESSING AND PACKAGING CONTAMINANTS

2915. Phthalate esters and the pregnant mouse

Singh, A. R., Lawrence, W. H. & Autian, J. (1974). Mutagenic and antifertility sensitivities of mice to di-2-ethylhexyl phthalate (DEHP) and dimethoxyethyl phthalate (DMEP). *Toxic. appl. Pharmac.* **29**, 35.

Phthalate plasticizers have been the subject of much experimental work since they were found to migrate into blood from PVC transfusion packs (*Cited in F.C.T.* 1971, **9**, 910; *ibid* 1973, **11**, 914). The group cited above have previously reported teratogenic effects due to the ip administration of eight phthalates to pregnant rats (*ibid* 1972, **10**, 724). Of those tested, dimethoxyethyl phthalate (DMEP) produced the most severe reactions, while the effects of di-2-ethylhexyl phthalate (DEHP) were relatively mild. The present report is concerned with dominant lethal mutation and antifertility studies on these two esters in mice.

LD_{50} values were determined in adult male Harlan/ICR mice weighing 25–30 g and groups of ten male animals were given the undiluted phthalate ip in a single dose equivalent to 33, 50 or 67% of the LD_{50}. Each male was caged with two virgin females, which were replaced weekly throughout a 12-wk experimental period. All male animals were checked for fertility before their use in the study. Day 0 of pregnancy was determined by the appearance of a vaginal plug, and the dams were then caged separately until day 13–17, when they were killed. The numbers of corpora lutea, implantations, early and late foetal deaths, pre-implantation losses and viable foetuses were recorded. The antifertility effect was measured as a reduction in the incidence of pregnancies, and the mutagenic effect was derived directly from the increase in the number of early foetal deaths and indirectly from the reduction in the total number of implantations.

DMEP was more acutely toxic than DEHP, with an LD_{50} of 3·57 ml/kg as opposed to 38·35 ml/kg. Four animals given the highest dose of DEHP died 2 wk after treatment and two given the highest dose of DMEP died within 2 hr. The latter animals were replaced. Both compounds caused a dose-related decrease in fertility and showed some mutagenic effect. A marked reduction in the incidence of pregnancy was associated with the highest dose of each compound and to some extent with the lower doses of DEHP. The mean number of implantations in each pregnancy was also reduced in the highest-dose groups. Injection of DEHP had a significant effect on the premeiotic stage of spermatogenesis, while DMEP affected the postmeiotic stage.

Early foetal death was affected significantly by DEHP during the last few weeks of the test period, the effect being inversely related to dosage. This suggests that low doses of DEHP enhance the incidence of early foetal deaths, while high doses in some way reduce it. Although the pregnancy rate for the group on the high DEHP dose was still lower than that of the controls, the numbers of implantations per pregnancy and litter sizes were almost identical during these last 4 wk, when fertilization would have been effected by sperm that were in the early premeiotic stage at the time of DEHP injection. This effect was not confined to any particular animals nor was it observed with DMEP, and the authors do not suggest any reason for the observation.

[The adverse reproductive and genetic effects found in this study seem to warrant further investigation in view of the widespread incidence of these compounds in the environment.]

THE CHEMICAL ENVIRONMENT

2916. More on chromium sensitization

Schneeberger, H.-W. u. Forck, G. (1974). Tierexperimentelle Chromallergie. Einfluss der Valenzen und der chemischen Umgebung der Chromionen. *Arch. dermatol. Forsch.* **249**, 71.

Chromium (Cr) can be absorbed through the skin in both valency forms, Cr^{VI} and Cr^{III}, and its absorption is enhanced by surfactants such as Triton X-100 and varies with pH (*Cited in F.C.T.* 1973, **11**, 507). These factors have a great bearing on the problem of Cr sensitization.

The paper cited above points to some more factors relevant to the question of Cr allergy. Aqueous solutions of various Cr^{VI} and Cr^{III} compounds, in concentrations of 5% with 2% Triton X-100, were applied to the shaved skin of guinea-pigs on ten occasions at intervals of 24 hr. Tests for sensitization were made by applying 0·5% solutions of the various compounds after an interval of 8 wk or more. The Cr^{VI} compounds (chromate and dichromate) elicited a high degree of sensitization. Tests with Cr^{III} compounds in the form of the hydrated chloride, a urea complex or the triethylenediamine chelate demonstrated a sensitizing capacity inversely proportional to the strength of Cr bonding in the complex, while water-insoluble Cr^{III} compounds showed only weak sensitizing potential.

2917. More data on children exposed to lead

Landrigan, P. J., Whitworth, R. H., Baloh, R. W., Staehling, N. W., Barthel, W. F. & Rosenblum, B. F. (1975). Neuropsychological dysfunction in children with chronic low-level lead absorption. *Lancet* **I**, 708.

A major issue in connexion with the possible effects of environmental lead on children is the question of whether blood levels of 40–80 μg/100 ml have an adverse effect on the nervous system (*Cited in F.C.T.* 1975, **13**, 280). Claims have been made in the past that blood-lead levels of this order might result in behavioural abnormalities, a slight impairment of voluntary muscle activity and weakness of peripheral nerves (*ibid* 1975, **13**, 281). The findings were not consistent, however, and in an attempt at further clarification, the authors cited above examined a group of 46 children between the ages of 3 and 15 yr who were symptom-free but who had blood-lead concentrations of 40–68 μg/100 ml and a second group of 78 comparable children with individual blood-lead levels below 40 μg/100 ml and a group mean of 27 μg/100 ml. All the children lived near a large lead-emitting ore-smelter in Texas.

Special intelligence tests designed to evaluate performance IQ indicated that the children with the high blood-lead levels performed less well than the children with the lower levels, but ratings of full-scale IQ, general behaviour and hyperactivity did not differ between the two groups. Clinically, the children with a high blood-lead level showed some weakness of the muscles of the forearm compared with the control group, a finding which may have been an indication of low-grade motor neuropathy.

2918. Tellurium on the brain

Agnew, W. F., Snyder, D. A. & Cheng, J. T. (1974) Metabolic inhibition of iodide transport in choroid plexus and ciliary body by tellurium and selenium. *Microvasc. Res.* **8**, 156.

It has been shown in rats that tellurium (Te) crosses the placental barrier and causes hydrocephalus in offspring, the phenomenon being associated with Te accumulation in the choroid plexus of the lateral and fourth ventricles of the brain (*Cited in F.C.T.* 1973, **11**, 922). In unpublished work mentioned in the paper cited above, prolonged feeding of Te to rats resulted in Te accumulation in the ciliary body of the eye.

The paper cited reports *in vitro* and *in vivo* experiments designed to show whether Te accumulation in these two areas is due to active transport and to compare the effects of Te and selenium (Se) on the active transport of iodide in the choroid plexus and ciliary body. When incubated for 1 hr with ^{127m}Te (as tellurite), the isolated ciliary body (dissected out with the iris) or choroid plexus of the rabbit showed a roughly fivefold concentration of Te compared with the content of the surrounding medium. This accumulation of Te was not inhibited by digitoxin or by thiocyanate, but iodoacetate, which blocks anaerobic glycolysis, significantly decreased the Te uptake of the ciliary body although not of the choroid plexus. *p*-Chloromercuribenzoate, which has been shown to block the binding of Te to protein, decreased Te uptake by both types of tissue. Both tellurite and selenite reduced the iodine-131 (^{131}I) uptake of these tissues significantly *in vitro*. The *in vivo* effect of intramuscular injections of finely powdered metallic Te or Se given in doses of 50 mg/kg twice weekly for 4 wk was also studied. Te injections inhibited ^{131}I transport by the ciliary body and choroid plexus, while Se injections did not.

These results indicate that Te accumulation depends mainly on protein-binding rather than on an active transport process. It appears that Te is enzymically active and is able to inhibit at least one energy-dependent transport system in the choroid plexus. This activity, combined with the ability of Te to cross the placental barrier and accumulate selectively in the foetal choroid plexus, provides some explanation for the hydrocephalus-inducing potential of Te.

2919. Chlorinated hydrocarbons and the liver

Traiger, G. J. & Plaa, (1974). Chlorinated hydrocarbon toxicity. Potentiation by isopropyl alcohol and acetone. *Archs envir. Hlth* **28**, 276.

It has already been shown that pretreatment of dogs with ethanol potentiates the hepatotoxicity of chloroform and carbon tetrachloride, but not that of trichloroethylene or 1,1,1-trichloroethane (*Cited in F.C.T.* 1968, **6**, 808). It appears, therefore, that ethanol potentiates the effects only of those chlorinated hydrocarbons that already possess marked hepatotoxicity.

Now the picture has been extended to cover the possible influence of isopropanol and its metabolite, acetone, on the hepatotoxicity of chlorinated solvents. Mice were given acetone or isopropanol in an orally intubated dose of 2·5 ml/kg (as a 25%, v/v, solution) 18 hr before receiving an ip injection of chloroform, trichloroethylene or 1,1,1- or 1,1,2-trichloroethane. The chlorinated hydrocarbons were given in corn oil in a range of doses around the threshold for induction of liver damage, as assessed by an increase in the activity of serum glutamic–pyruvic transaminase (SGPT).

The known hepatotoxicities of the chlorinated hydrocarbons tested decrease in the following order: chloroform > 1,1,2-trichloroethane > trichloroethylene > 1,1,1-trichloroethane. The augmentation of hepatotoxicity by prior treatment with acetone or isopropanol correlated with this ranking, potentiation being maximal with chloroform and negligible with

1,1,1-trichloroethane. Both acetone and isopropanol potentiated the hepatotoxicity of doses of 1,1,2-trichloroethane that, when administered alone, had no effect on SGPT activity. With trichloroethylene, a dose of 1·5 mg/kg was required to demonstrate any marked potentiation by isopropanol.

Preliminary studies indicate that similar effects are exerted by inhaled acetone or isopropanol, both of which are commonly encountered industrial solvents, but while further work on this aspect is clearly required, the current study suggests that the likelihood of hazardous interaction between these two types of solvent should be largely predictable on the basis of the hepatotoxic potential of the halogenated hydrocarbon.

2920. Hepatitis in workers exposed to diaminodiphenylmethane

McGill, D. B. & Motto, J. D. (1974). An industrial outbreak of toxic hepatitis due to methylenedianiline. *New Engl. J. Med.* **291**, 278.

The hepatotoxicity of 4,4′-diaminodiphenylmethane (DDM) has been demonstrated in a variety of animal species (*Cited in F.C.T.* 1967, **5**, 115; *ibid* 1975, **13**, 480) and, following the accidental contamination of a bag of flour, also in man (*ibid* 1967, **5**, 120). However, the paper cited above apparently provides the first report of liver damage resulting from industrial handling.

Between 1966 and 1972, 12 cases of hepatitis were identified among workers in a large manufacturing firm. All the cases involved men employed in folding and kneading with cloth-gloved hands a hot epoxy-resin mixture containing 10% DDM. The mixture was subsequently cooled and pulverized into a fine powder which settled rapidly on workers and their surroundings, but the provision of masks and exhaust ventilation was thought to obviate any inhalation hazard. The atmospheric concentration of DDM in 1969, checked after the first three cases of hepatitis had been reported, was 0·1 ppm, and in 1971 (after diagnosis of a further five cases) this was lowered to 0·018 ppm by the installation of more exhaust vents. However, this and other measures, including partial enclosure of the work area, daily clean overalls and showers, failed to prevent another two cases. The atmospheric concentration of DDM was subsequently lowered to 0·0064 ppm and the wearing of protective helmets with separate air supplies became mandatory, but in spite of this two further cases occurred. At this stage, oral and dermal treatment of rabbits with floor sweepings from the mill provided further evidence that DDM was the cause of the hepatitis. Sweepings collected after addition of DDM to the reaction mixture produced clear evidence of hepatotoxicity in the animals, while those collected earlier and shown to be free of DDM gave negative results. Only by the introduction of complete air-supplied PVC suits with attached rubber gloves, and the subsequent development of a fully-enclosed automated blending system, were further cases finally prevented.

Symptoms displayed by the victims included weakness, jaundice and dark urine, accompanied in the majority by abdominal pain, nausea and vomiting and sometimes also by headache, acholic stools, pruritus, rashes and pains in the muscles and joints. Biochemical evidence of liver damage was provided by greatly increased serum levels of bilirubin, glutamic–oxalacetic transaminase and alkaline phosphatase. All those affected became ill within 1–18 days of working intensively with DDM and recovered within 7 wk. Similar symptoms were displayed by a worker in another company, temporarily contracted to pulverize DDM. Of 21 other similarly exposed workers who were interviewed, four recalled symptoms apparently consistent with hepatitis. The gloves of the men handling the resin mixture always became ragged, holey and saturated with the material, and it appeared likely that the chief route of entry for DDM was through the skin of the hands. When re-examined between 9 months and 5·5 yr later, none of these hepatitis patients showed any clinical or biochemical evidence of chronic liver disease.

2921. Carcinogenesis by 3,3′-dichloro-4,4′-diaminodiphenyl ether

Herrmann, I. F., Schauer, A. u. Kamke, W. (1973). Morphologische und histochemische Untersuchungen während der Cancerogenese des äusseren Gehörganges der Ratte, induziert durch 3.3′Dichlor-4,4′diaminodiphenyläther. *Arch. Oto-Rhino-Lar.* **206**, 11.

The possibility that administration of 3,3′-dichloro-4,4′-diaminodiphenylether (DDDE) to rats might induce carcinoma of the auditory canal was first suggested by Steinhoff & Grundmann (*Naturwissenschaften* 1970, **57**, 676).

The present paper describes the appearance of multiple lesions of the epithelium of the auditory canal and/or deep ceruminal gland in rats after administration of 400 mg DDDE/kg/wk for a total period of 200–340 days by sc injection into the back. In all the animals undergoing this treatment, these tissues showed sharply defined areas of high alkaline-phosphatase activity and reduced lactate-dehydrogenase and NADH-dehydrogenase activity, compared with the same regions in untreated animals or with the regenerating epithelium of rats in which the superficial tissue of the auditory canal and gland had been deliberately damaged. 5-Bromoindoxyl-acetate esterase activity, which is characteristically high in sebaceous-gland tissue, was lost in the same areas in the DDDE-treated animals. After 310 days, foci of epithelial hyperplasia and a high incidence of papillomas and adenomas were evident in the DDDE-affected areas and after 390 days four of the treated animals showed papillary carcinomas, squamous-cell carcinomas and carcinomas of the ceruminal gland.

The enzyme changes detected in this study in the early stages of ear-duct and sebaceous-gland carcinogenesis are similar to those that have been shown to occur in the early stages of neoplasia of the nasal mucous membranes.

2922. Teratogenic approach to DES problem

Stafl, A. & Mattingly, R. F. (1974). Vaginal adenosis: A precancerous lesion? *Am. J. Obstet. Gynec.* **120**, 666.

An interesting view has recently been put forward in connexion with the occurrence of vaginal adenocarcinoma in young girls exposed prenatally to diethylstilboestrol (DES).

The administration of DES during pregnancy interferes with the normal development of the embryonic vagina so that the columnar epithelium, which is usually limited to the uterine cavity and cervical canal, extends into a large area of the vagina, where it replaces the normal squamous epithelium. The presence of columnar epithelium in the vagina is, clinically, called adenosis. In the vagina, the columnar epithelium is exposed to an abnormal environment, particularly in respect of the low pH which is normally prevalent in that organ. Under these abnormal conditions, irregular patches of this columnar epithelium undergo squamous metaplasia, a process that has been found in a high percentage of girls under the age of 25 yr in whom adenosis has followed DES exposure *in utero*. The process of transition from the columnar to the squamous form is thought to represent a stage of particular susceptibility to neoplastic transformation, in which some environmental mutagenic agent, such as a virus, may participate. The tumours that may arise at this stage are vaginal adenocarcinomas.

The areas of squamous metaplasia probably also represent sites where invasive squamous carcinomas are liable to develop in later years, since in a study of 131 cases of vaginal adenosis in girls exposed to DES *in utero*, two cases of non-invasive squamous carcinoma and five cases of abnormal epithelial thickening (dysplasia) were diagnosed. These lesions are usually regarded as precursors of frank malignancy.

The authors consider the biological effect of DES to be one of teratogenesis, rather than carcinogenesis, since the compound induces congenital alterations in the vaginal epithelium where neoplastic transformation can occur at a later age through some agency other than DES.

[This conclusion is of major importance in connexion with the assessment of the carcinogenic hazard of diethylstilboestrol.]

2923. Formaldehyde dermatitis in fashion

Schorr, W. F., Keran, E. & Plotka, E. (1974). Formaldehyde allergy. The quantitative analysis of American clothing for free formaldehyde and its relevance in clinical practice. *Archs Derm.* **110,** 73.

The finishing of certain textiles with formaldehyde resins to increase their crease-resistance has long been known to result in free formaldehyde (HCHO) residues, which can be removed by washing but which may again be generated by contact of the finishing resin with the wearer's body (*Cited in F.C.T.* 1966, **4,** 86). Although the quantities of HCHO thus produced are harmless to most people, those with an established allergy to HCHO may risk perpetuating their condition when they wear treated garments. According to US textile and garment manufacturers, fabrics sold there contain no more than 750 ppm HCHO, a concentration which failed to induce dermatitis in a group of persons known to be sensitive to HCHO (*ibid* 1963, **1,** 300).

The paper cited above describes the determination of free HCHO in 112 samples of fabrics from American textile manufacturers and distributors. All contained some free HCHO, the concentrations ranging from 1 to 3517 ppm. Fifteen samples of 100% polyester knit and eight samples of 100% polyacrylonitrile (Orlon) contained less free HCHO than was found in 100% rayon, 100% cotton, a 50% polyester–50% cotton mix and a 65% polyester (Dacron)–35% cotton mix. The HCHO levels in both the 100% polyester and 100% polyacrylonitrile fabrics were below 30 ppm. Rayon on the other hand varied remarkably, one sample containing 3517 ppm HCHO and another only 15 ppm. The authors noted a sharp discrepancy between their findings and previous data derived from the manufacturers. In view of this situation, it seems that HCHO in clothing must be taken into account as a possible cause of dermatitis when clinical investigations of an allergy are being carried out.

2924. Assessing exposure to PTFE

Polakoff, P. L., Busch, K. A. & Okawa, M. T. (1974). Urinary fluoride levels in polytetrafluoroethylene fabricators. *Am. ind. Hyg. Ass. J.* **35,** 99.

Toxic breakdown products of polytetrafluoroethylene (PTFE) become significant only at temperatures exceeding those reached in normal cooking procedures (*Cited in F.C.T.* 1974, **12,** 794). One pyrolysis product of PTFE, carbonyl fluoride, formed at about 400°C and encountered in industrial processes, is excreted in the urine as inorganic fluoride ion, which is therefore suggested as an index of exposure to PTFE products.

Human exposure to PTFE was studied at a factory employing 130 persons, in which over a 5-yr period repeated fever attacks attributed to polymer fumes had been reported. Breathing-zone samples of air and samples from the general working area showed concentrations of polymer dust ranging from 0·4 to 5·5 and from 0 to 3·2 mg/m^3 respectively. Of the 77 individuals who completed a medical questionnaire, 60 out of a total of 70 production workers stated that they had experienced polymer fume fever, half of them during the previous year. Ten reported more than three fever episodes during the previous year. Fluoride-ion levels in urine samples ranged from 0·098 to 2·19 mg/litre, a level below that at which systemic manifestations would be expected. The level in the local water supply was 0·19 mg/litre. Statistical analysis indicated that the mean urinary fluoride-ion excretion among workers exposed to PTFE for 1 yr or more and having a history of polymer fume fever was significantly higher than that among workers with shorter exposures and no history of fever. There was no evidence that exposure for longer than 1 yr with additional episodes of fever was paralleled by any further rise in urinary excretion of fluoride ion.

2925. Hexane and the liver microsomes

Krämer, A., Staudinger, Hj. & Ullrich, V. (1974). Effect of *n*-hexane inhalation on the monooxygenase system in mice liver microsomes. *Chemico-Biol. Interactions* **8,** 11.

n-Hexane has recently been rated as a moderate hepatotoxin on the basis of the serum levels of ornithine carbamoyltransferase recorded after its injection into guinea-pigs (*Cited in F.C.T.* 1975, **13,** 408). When inhaled by the rat, it accumulates in the tissues in direct proportion to their lipid content, and levels in

the liver rise as triglycerides accumulate in that organ (*ibid* 1974, **12**, 431). *n*-Hexane is hydroxylated by the monooxygenase system of the hepatic microsomes, in common with many other lipophilic organic compounds, and like them might be expected to enhance this microsomal activity. However, no such enhancement was found in preliminary tests by the authors cited above, using a single oral or ip dose of *n*-hexane, and far more severe conditions were therefore used in the present study.

When mice were exposed for 23 hr daily for up to 4 days to an atmosphere of 2·5–3·0% *n*-hexane, body weight decreased slightly and there was a marked increase in liver weight (expressed in relation to body weight) after 20–30 hr. This was accompanied by an even greater increase in the microsomal–protein content of the liver. In the early stages this could be accounted for largely by rapid increases in the specific activities of cytochrome *P*-450 and the corresponding NADPH-dependent reductase, both of which reached a maximum (relative to microsomal-protein weight) at 48 hr and declined slightly thereafter. Cytochrome b_5 levels began to rise only after 24 hr, and continued to increase sharply throughout the remaining 3 days. Using cyclohexane as a substrate, it was shown that microsomal hydroxylation activity also increased, in parallel with the cyclohexane binding difference spectrum; the latter reflected the formation of a cyclohexane–cytochrome *P*-450 complex, which is an essential requirement for the monooxygenase reaction. However, there was no strict correlation between the cyclohexane-hydroxylation activity and cytochrome *P*-450 content. The effects of this high concentration of *n*-hexane were similar throughout to those obtained with phenobarbitone. Pretreatment with either compound enhances hydroxylation in the ω-1 position, in contrast to benzo[*a*]pyrene treatment, for example, which mainly increases 3-hydroxylation. These findings suggest that exposure to *n*-hexane brings about a qualitative alteration in the cytochrome *P*-450 species.

2926. A breath of solvent

Nomiyama, K. & Nomiyama, H. (1974). Respiratory retention, uptake and excretion of organic solvents in man. Benzene, toluene, *n*-hexane, trichloroethylene, acetone, ethyl acetate and ethyl alcohol. *Int. Arch. Arbeitsmed.* **32**, 75.

Nomiyama, K. & Nomiyama, H. (1974). Respiratory elimination of organic solvents in man. Benzene, toluene, *n*-hexane, trichloroethylene, acetone, ethyl acetate and ethyl alcohol. *Int. Arch. Arbeitsmed.* **32**, 85.

Studies of the absorption and excretion of inhaled solvents are generally undertaken with a view to establishing reasonably safe industrial limits of exposure. Among solvents subjected recently to relevant studies, for this or other reasons, are acetone (*Cited in F.C.T.* 1975, **13**, 151), trichloroethylene (*ibid* 1973, **11**, 1154; *ibid* 1974, **12**, 163) and *n*-hexane (*ibid* 1974, **12**, 431). There is much scope for further investigation into the fate in man of relatively low environmental concentrations of organic solvents, since (in the case of acetone for instance) there is some evidence that cumulation may be hazardous.

The first study cited above describes the exposure of male and female volunteers to benzene (52–62 ppm), toluene (98–130 ppm), *n*-hexane (87–122 ppm), acetone (127–131 ppm), ethyl acetate (94–137 ppm) or ethanol (103–140 ppm) for 4 hr or to trichloroethylene (252–380 ppm) for 2·7 hr. At hourly intervals, samples of expired air were collected and retention of the gas was estimated. A roughly constant retention level was reached after 2 hr with all the solvents except benzene, with which stabilization took some 3 hr. At 2 hr, retention was lowest with *n*-hexane, followed in increasing order by acetone and ethanol. Ethyl acetate, trichloroethylene, toluene and benzene showed a relatively high degree of retention. Retention of acetone was lower in women than in men, but other solvents showed no marked sex difference. Immediately after exposure ceased, the ratios of excretion to uptake were similar for all the solvents except ethyl acetate, the excretion ratio of which was only some 14–17% of that of the other solvents. The percentage uptake of the solvents varied from 28 for hexane to 60 for ethyl acetate, and the percentage excretion from 3 for ethyl acetate to 22 for hexane.

The second study cited, a continuation of the first, showed that after cessation of solvent exposure there was a rapid decrease in the concentration in expired air. Toluene, trichloroethylene and acetone were less rapidly eliminated via the lungs by women than by men, a reflection of the lower blood concentrations of these solvents in women. A close relationship between the environmental (exposure) level and the respiratory concentration was demonstrable for several hours after the end of exposure. This relationship enables the degree of exposure to be assessed from the respiratory gas concentration when both retention and elimination are sufficiently pronounced. One interfering factor is the metabolism of the solvent, which is considerable for ethyl acetate and substantial also for ethanol. Large amounts of acetone, benzene and *n*-hexane, on the other hand, were eliminated through the lungs unchanged. It is suggested that the determination of respiratory elimination rates may prove useful for estimating industrial exposure to benzene, toluene, acetone and trichloroethylene.

NATURAL PRODUCTS

2927. The non-hepatotoxicity of congeners

Hillbom, M. E., Franssila, K. & Forsander, O. A. (1974). Effects of chronic ingestion of some lower aliphatic alcohols in rats. *Res. Commun. chem. Path. Pharmac.* **9**, 177.

The congeners present in alcoholic beverages include *n*-propanol, isobutanol and isoamyl alcohol. These compounds may enhance the neuropharmacological effects of ethanol (*Cited in F.C.T.* 1968, **6**, 430) and it has also been suggested that they may contri-

bute to the production of fatty liver in chronic alcoholism (*ibid* 1970. **8.** 435). a hypothesis supported by the greater incidence of alcohol-induced liver damage in countries where high-congener beverages. such as brandy. whisky and wine are consumed (Snapper. in *Alcohol and the Liver*. edited by W. Gerok. K. Sickinger and H. H. Hennekensen, p. 449. F. K. Schattauer. Stuttgart. 1971). However. other studies have failed to reveal any effect of the congeners on a variety of parameters. including liver function and hepatic lipid and triglyceride levels (*Cited in F.C.T.* 1965, **3.** 639: Gaillard & Derache, *Fd Cosmet. Toxicol.* 1966, **4.** 515). and there was no evidence of hepatotoxicity in rats fed isoamyl alcohol at intake levels of up to 1 g/kg/day for 17 wk (Carpanini *et al. ibid* 1973, **11.** 713).

In the study cited above. the possibility that some congeners might be more hepatotoxic than ethanol was tested by providing rats with a 1 M solution of ethanol. *n*-propanol or isobutanol as their sole drinking fluid for 4 months. However. the only evidence of hepatotoxicity was a slight increase in the fat content of the livers of some of the ethanol-treated rats, and even this was unaccompanied by fatty. cirrhotic or fibrotic lesions. Other effects seen included a decrease in weight gain and in efficiency of food utilization in rats given *n*-propanol. while isobutanol-treated rats increased their fluid intake and were found at autopsy to have enlarged gas- or food-filled stomachs, accompanied in some cases by constipation and signs of bleeding in the small intestine. The rats in all cases were on a nutritionally adequate diet and it is possible that a diet of poorer quality, or administration of the test fluid for longer periods, might have produced different results.

2928. Monocrotaline and the heart

Chesney. C. F., Allen, J. R. & Hsu, I. C. (1974). Right ventricular hypertrophy in monocrotaline pyrrole treated rats. *Expl mol. Path.* **20.** 257.

Monocrotaline is a pyrrolizidine alkaloid present in *Crotalaria spectabilis*, various Senecio species and some other closely related plants. The liver lesions produced by compounds of this type in many animal species are considered to be the direct result of the conversion of the native alkaloids into pyrrole derivatives by microsomal enzyme systems (*Cited in F.C.T.* 1974. **12.** 559; *ibid* 1975. **13.** 158).

Investigations described in the paper cited above show that animals given an iv injection of monocrotaline pyrrole (MCP) in sublethal doses develop hypertensive pulmonary vascular lesions and cor pulmonale without any appearance of hepatic damage. Rats were given a single tail-vein injection of 2 or 4 mg MCP/kg and 4 wk later the systolic blood pressure was determined within the right ventricle. under urethane anaesthesia. and blood and organ samples were examined. Peak systolic and end diastolic pressures in the right ventricle were significantly raised by MCP. The right side of the heart was seen to be markedly dilated and hypertrophied. with a thickened and pale right ventricular wall. The pleural cavity contained cloudy yellow fluid, and the lungs were congested, haemorrhagic and oedematous. Within the lungs. atelectasis. proliferation of fibrous tissue and an abundance of macrophages. pneumocytes and extravasated blood components appeared. The thickened and occluded pulmonary arteries and arterioles showed hypertrophy of muscular components and enlargement of the endothelial lining cells. The liver and spleen were moderately congested.

The findings indicate that MCP acts primarily upon the endothelium of the pulmonary capillary bed. Firm binding of MCP to the endothelial cells at the time of injection seems likely, followed within 24 hr by injury which markedly increases capillary permeability. fibrin and thrombus accumulation and pulmonary oedema. Impedance of the pulmonary circulation by oedema raises the blood pressure and in turn provokes further pulmonary endothelial changes. Eventually the sustained pulmonary hypertension induces right-ventricle hypertrophy which progresses to heart failure.

2929. Navy beans and jack beans, raw or cooked

Jayne-Williams, D. J. & Burgess, C. D. (1974). Further observations on the toxicity of navy beans (*Phaseolus vulgaris*) for Japanese quail (*Coturnix coturnix japonica*). *J. appl. Bact.* **37.** 149.

It has been shown that the growth-inhibiting factor present in raw navy beans is not the trypsin inhibitor that is also present (*Cited in F.C.T.* 1966. **4.** 356). A diet containing raw navy beans has since been found to be lethal to conventional quail but only to depress the growth of germ-free birds (*ibid* 1974. **12.** 432). The toxicity was eliminated by autoclaving the beans. A phytohaemagglutinin was suggested as a possible factor in this toxic effect and it was shown that concanavalin A, a compound of this type isolated from jack beans, was toxic to quail. The study cited above provides further evidence that a similar factor operates in navy-bean toxicity.

Quail were fed diets containing up to 50% raw navy beans (RNB) or autoclaved bean meal (ANB). The lethal and growth-depressing effects diminished with a reduction in the proportion of RNB fed. RNB fermented with coliform organisms and sterilized before being fed to germ-free quail was non-lethal, while germinated RNB and lipid-extracted RNB were both lethal. Addition of dialysable constituents of RNB to a harmless diet did not render it toxic. The removal of carbohydrates soluble in 70% ethanol or susceptible to breakdown by bacteria did not influence the toxicity of RNB meal. Similarly, breakdown of proteins by pepsin or bacteria failed to remove the toxicity. Addition of hydrolysed casein to the RNB diet did not reduce the RNB toxicity. The toxic factor therefore appeared to be a protein which resisted proteolysis but was destroyed by autoclaving at 121°C for 15 min. It was also soluble at pH 3 and was precipitated by saturation with ammonium sulphate. These results indicate that a phytohaemagglutinin was responsible for the toxic effects of RNB as well as of the jack bean. It seems possible that its mode of action may be to impair body defence mechanisms that normally prevent tissue invasion by regular components of the intestinal microflora.

2930. Mustard oil capers

Mitchell, J. C. (1974). Contact dermatitis from plants of the caper family, Capparidaceae. Effects on the skin of some plants which yield *iso*thiocyanates. *Br. J. Derm.* **91**, 13.

Plants of the caper family (Capparidaceae) are the tropical relatives of the Cruciferae of temperate regions. They contain isothiocyanates, or glycosides that are converted to isothiocyanates by the action of the enzyme myrosinase. One such glycoside is glucocapparin, which gives rise to methyl isothiocyanate. The same enzyme converts siningrin, the glycoside of seeds of mustard (*Brassica nigra*), to allyl isothiocyanate.

Isothiocyanates may be primary irritants, inducing a chemical dermatitis, and in some individuals they may provoke allergic contact dermatitis into the bargain. The plant genera that possess irritant or sensitizing properties include Capparis, Cleome, Courbonia, Crateva and Gynandropsis. Some of these, especially species of Cleome (spider-flowers), are cultivated as ornamental plants and may prove troublesome to gardeners. Although the glycosides that give rise to the irritant isothiocyanates are mainly present in the seeds, they occur also in the green parts of some species. It appears that a 'substantial number' of garden plants and vegetables may be potential irritants if not potential sensitizers, and the author makes the point that, in view of the wide confusion that exists in connexion with the common names given to capparidaceous plants, it is important that in records of episodes of dermatitis the accurate botanical nomenclature be used.

2931. Pepper and the duodenum

Nopanitaya, W. & Nye, S. W. (1974). Duodenal mucosal response to the pungent principle of hot pepper (capsaicin) in the rat: Light and electron microscopic study. *Toxic. appl. Pharmac.* **30**, 149.

Hot green and red peppers are commonly used as food spices. Hot red peppers contain 0·1–1·0% of the pungent principle, capsaicin, which is unaffected by drying and is readily soluble in hot water. Investigations into the potential hepatocarcinogenicity of red peppers in man have given inconclusive results (*Cited in F.C.T.* 1970, **8**, 84). The study cited above describes changes induced in the duodenal lining of rats by contact with 10% capsicum in normal saline or a roughly equivalent solution of 0·014% synthetic capsaicin for various periods.

These solutions, in a volume of 0·8 ml, were instilled into the ligated upper duodenum of anaesthetized rats and retained therein for 2 min. Other lightly anaesthetized rats were given 2 ml of one or other solution by gastric intubation and were killed for examination after intervals of 6–60 min. This treatment provided a dose of 1 mg capsaicin/kg. All control animals received an appropriate volume of normal saline. Throughout the study, there was virtually no difference between the morphological changes induced by 10% capsicum or 0·014% capsaicin solution when the duration of exposure and route of administration were the same. Microscopic findings in the duodenal villi of animals in the 2-min test were similar to those in animals treated intragastrically and examined after 6 and 15 min, and were less severe than those seen after 30 min. The villi were oedematous, showing capillary congestion in the lamina propria and some lymphocyte infiltration, particularly after 15 min, when some epithelium had separated from the basement membrane around the tips of the villi. Villous-tip cells showed an increase in numbers of pyknotic nuclei. Similar but more pronounced changes appeared after 30 min while after 45 and 60 min many areas of the duodenal mucosa were denuded of epithelium and the remaining epithelial cells showed acidophilic cytoplasm and irregular small nuclei with distinct nuclear membranes.

Ultrastructural changes revealed mitochondrial swelling 2 min after intraduodenal instillation, together with a rarefied matrix and disorganized cristae. The numbers of free ribosomes and lysosomes increased, the endoplasmic reticulum was slightly extended and Golgi complexes were swollen. Nuclei showed some shrinking, and chromatin was clumped and concentrated at the inner periphery of the nuclear envelope. Less severe changes followed intragastric instillation of capsaicin for 6 min, but at 15 min the changes were similar in degree to those after intraduodenal instillation for 2 min. The severity of the changes was increased at 30 min and became maximal at 45 and 60 min.

The findings of this study indicate clearly that capsaicin, whether natural or synthetic, has a direct toxic effect on the duodenal cells of rats. The physiological consequences, which are of prime importance in man, require further investigation.

2932. Tannic acid in the enema

Eshchar, J. & Friedman, G. (1974). Acute hepatotoxicity of tannic acid added to barium enemas. *Am. J. dig. Dis.* **19**, 825.

Much discussion and debate on the possible hepatotoxicity of tannic acid in barium sulphate enemas has led to the tentative conclusion that, if unduly long retention is avoided and patients with extensive colonic lesions are not submitted to such treatment, the hazard of absorption can be kept within reasonable limits (*Cited in F.C.T.* 1967, **5**, 244). Similar conclusions were reached in the recent study cited above.

In 50 patients who had shown no prior indications of liver damage and in 11 known to have some liver damage, liver-function tests were performed before and after the administration of a 1500 ml enema of barium sulphate to which 1% tannic acid had been added to improve colonic visualization. Three patients in the first group showed minor rises in the serum activity of glutamic–oxalacetic and glutamic–pyruvic transaminases after the enema; in one the change was attributed to coronary ischaemia, in the second to retention of the enema for longer than 30 min, and in the third to childhood susceptibility. Four patients in the second group showed some deterioration in liver function after the enema, as demonstrated by the bromsulphthalein retention test (three patients) or by determination of alkaline phosphatase (one patient).

There is thus no evidence from this study that the addition of 1% tannic acid to a barium enema is likely

to cause liver damage in an adult who is free from prior liver dysfunction, who is not subject to retarded bowel evacuation, and who has no severe and extensive colonic lesions. However, further evaluation of the hazard to children and to adults with liver dysfunction is considered necessary.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2933. Lauryl sulphates and the skin

Tovell, P. W. A., Weaver, A. C., Hope, J. & Sprott, W. E. (1974). The action of sodium lauryl sulphate on rat skin—an ultrastructural study. *Br. J. Derm.* **90**, 501.

Gloxhuber, Ch., Potokar, M., Braig, S., van Raay, H. G. u. Schwarz, G. (1974). Untersuchungen über das Vorkommen eines sensibilisierenden Bestandteils in einem technischen Alkyläthersulfat. *Fette Seifen AnstrMittel* **76**, 126.

Sodium lauryl sulphate (SLS) is an established skin irritant, and dermatitis has been reported after the application under occlusion of hydrophilic ointments containing it (*Cited in F.C.T.* 1974, **12**, 798). The paper by Tovell *et al.*, cited above, describes ultrastructural alterations in rat skin washed six times in 3 days with 1% aqueous SLS. Coarsening, reddening and oedema resulted. Electron-microscopic examination of the epidermis showed lipid deposits in most cells, marked spongiosis and vesiculation, partial detachment of basal cells with rupture of the basal lamina, condensation of tonofibrils within cells of the basal-cell and prickle-cell layers, deposition of protein between the deeper epidermal cells to produce oedema and blistering, and thickening of the epidermis and stratum corneum. The numbers of keratohyalin granules were reduced but their size and structure were unaltered. In general, the picture was one of low-grade irritation with accelerated keratinization.

Allergic reactions have been reported in Scandinavia to have followed the handling of a dish-washing liquid containing sodium or ammonium lauryl ether sulphate (LES) of the formula $H(CH_2)_n.(OC_2H_4)_m.OSO_3^-$, where n = 12–14 and m = 2–4 (Magnusson & Gilje, *Acta derm.-vener., Stockh.* 1973, **53**, 1). Gloxhuber *et al.* (second paper cited above) fractionated various commercial samples of LES, including that implicated in the Scandinavian reactions, and tested the individual fractions in guinea-pigs in an attempt to identify the sensitizing factor. This was found to be extracted in petroleum ether and iso-octane/acetic acid. The sensitizing fraction consisted mainly of a fatty alcohol ethoxylate containing 2 mols ethylene oxide, and it also contained carbonyl groups derived from ketones or esters. The sensitizing component was not present in all samples of LES examined, and was attributed to exceptional manufacturing conditions under which ethoxylation was carried out at an unusually high temperature.

Fd Cosmet. Toxicol. Vol. 13, pp. 595–600. Pergamon Press 1975. Printed in Great Britain

LETTERS TO THE EDITOR

L-GLUTAMIC AND L-ASPARTIC ACIDS—A QUESTION OF HAZARD?

Sir,—In a recent letter to this Journal, Bigwood (*Fd Cosmet. Toxicol.* 1975, **13**, 300) advanced the *a priori* argument that monosodium glutamate (MSG) and aspartate (MSA) cannot be toxic at the doses reported by us because humans normally ingest even larger amounts of these amino acids (as part of food protein) on a regular basis. The major flaw in this argument is that it totally ignores the all-important difference between ingesting a free amino acid and ingesting an amino acid bound in protein. In the former case, absorption of the entire amino acid load is immediate and this leads to much higher peaking of the amino acid level in the blood than if a similar amount were released slowly over a matter of hours by the digestive process. There is evidence that the gut normally transaminates MSG and MSA to alanine, an amino acid that does not have excitotoxic potential, and this, together with paced release by digestion, may represent a protective mechanism to prevent elevated blood levels of these neurotoxic amino acids.

Bigwood (*loc. cit.*) complains that my use of a gastric tube to introduce MSG into the stomach of infant animals is unnatural, without pausing to realize that the introduction of free MSG into foods in concentrations up to 0·6% (in addition to the MSG contained in the food protein) is also unnatural. This is a higher dose of free MSG than is ordinarily encountered in natural foods and ingesting it as free MSG may allow it to circumvent the gut's transamination mechanism, thus permitting a disproportionately high percentage of it to enter blood as glutamate rather than as alanine. Incidentally, this complaint that we intubate large volumes of fluid into infant animals is incorrect. We use microlitre syringes in experiments on infant rodents and introduce volumes usually in the range of 50–100 μl. In the case of infant monkeys, we dissolved our MSG in dilute skim milk and limited the total volume to 7–10 ml (Olney *et al. J. Neuropath. exp. Neurol.* 1972, **31**, 464).

Prof. Bigwood's armchair logic—that MSG or MSA cannot be toxic at 0·5–2 g/kg because this much is ingested in protein every day—is simply irreconcilable with laboratory evidence. We have documented quite clearly that these amino acids do destroy hypothalamic neurons at these levels of oral intake. Our initial MSG findings were corroborated in infant mice and rats in an early report from a second laboratory (Burde, Schainker & Kayes, *Nature, Lond.* 1971, **233**, 58) and more recently in a lengthy neuropathological report on both infant and adult mice from a third laboratory (Lemkey-Johnston & Reynolds, *J. Neuropath. exp. Neurol.* 1974, **33**, 74). Our observation of brain damage from MSG administered orally at 0·5–2 g/kg in animals of various ages does, indeed, suggest that this common constituent of food protein could be toxic for young humans at intake levels close to those encountered from natural sources. Instead of approaching the issue like authorities of an earlier age who knew the world to be flat, nutritionists would be well advised to begin questioning the use of this neurotoxic compound as an additive to children's food. Very little is known about the capacity of immature humans to metabolize either MSG or MSA in the gut or elsewhere or about individual variations which might lead to abnormally high blood levels of MSG or MSA from relatively small amounts added as free amino acids to the diet. In experimental animals, MSG destroys hypothalamic neurons silently, i.e. without the animal showing observable signals of distress. When the integrity of the developing human nervous system is at stake, I respectfully submit that it is much better to be safe than sorry.

Bigwood (*loc. cit.*) also asserts that our findings must be regarded as species-specific. Since every animal species we have studied (mice, rats, rabbits, chicks, guinea-pigs and rhesus monkeys) sustained lesions from MSG treatment, whereas no such neuropathology appeared in control brains, we believe we are dealing with a species-general phenomenon. However, our demonstration of MSG-induced brain damage in immature monkeys has been vigorously challenged. For the challenger's point of view, see Golberg *et al.* (*New Engl. J. Med.* 1974, **290**, 1326) and for our discussion of species specificity, see Olney *et al.* (*ibid* 1973, **289**, 1374).

Regarding aspartame, which is 45% MSA, Bigwood (*loc. cit.*) makes numerous averaging-out computations intended to show that very little MSA will be added to the diet by this new sweetener. The problem is that we are dealing with an acute neurotoxic mechanism—one which is triggered by a single instance of excessive intake. Putting it another way, a single elevation of blood MSG or MSA above a certain toxic threshold is all that is required for hypothalamic neurons to be destroyed. By focusing on the average intake of aspartame per capita rather than considering the maximal amount which some sweets-loving children might consume, Prof. Bigwood is merely circumnavigating the problem. The FDA has estimated the maximal daily intake of aspartame to be 1·7 g. Prof. Bigwood will have to abandon the 'averaging-out' framework and recalculate according to 'maximal intake' estimations if he wishes to grapple realistically with this problem.

Finally, it appears that he totally misses the gist of my concern over aspartame. My concern is that MSG as used today in children's foods leaves only a slim margin of safety (perhaps five- to tenfold) for children at maximal consumption rates, and that the MSA content of aspartame may further reduce this slim margin by virtue of MSA and MSG acting by an additive toxic mechanism. The only way to approach such a problem is to study the combined toxic potential of these compounds. I assume Prof. Bigwood agrees with me that such studies are needed.

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MOUSE DATA

Sir,—A report by Walker *et al.* (*Fd Cosmet. Toxicol.* 1973, **11**, 415) on the long-term toxicity of dieldrin in mice concluded that exposure to the compound at various levels in the range 0·1–10·0 ppm in the diet for periods up to 2 yr resulted in liver lesions, including an increased incidence of liver parenchymal-cell tumours. In the course of a re-evaluation of the basic data for the purpose of carrying out a relative risk analysis with respect to tumour development, a number of transcription errors were detected in Table 1 of the published paper.

A corrected Table 1 is reproduced on p. 598. The principal change is in the groups of females given 0·1 and 1·0 ppm dieldrin, the number of mice in these two groups having originally been reported as being 30 less than was actually the case. The tumour counts, while correct, were expressed as a percentage of the misreported group size and consequently the true percentages for these groups are approximately one quarter less than the previously published figures. A further error was discovered in the lymphoid tumour incidence for males given the 10 ppm level: in this figure a decimal point was misplaced, so that the reported percentage was too great by a factor of ten.

The risk analysis, with respect to tumour development, considers not only the incidence but also the survival time prior to the observation of tumours. An essential part of this analysis, however, is the determination of how death occurred. If a specific tumour is considered responsible for death, then it is correct to use the survival time directly in the analysis; if the tumour is incidental to the death, then the survival time should be used only to form groups of animals who died at a similar time. For a discussion of the subtlety of this distinction and of the bias that can be introduced by performing the wrong type of analysis, the reader is referred to an editorial in the *British Journal of Cancer* (1974, **29**, 101).

A particular problem with this mouse study was that it was performed before it was appreciated that there was a need for such a precise tumour classification. Accordingly, the tumours were analysed initially as if they were responsible for the animal's death, bearing in mind that when an increased risk was shown this could be due to a false assumption about the nature of the tumour. In such situations a further analysis was carried out on the assumption that the tumour was not responsible for death. If both analyses gave positive results, it was apparent that there was a real increase in risk, despite the complexities introduced by the pattern of deaths. In this respect it was helpful to have the 4-amino-2,3-dimethylazobenzene (ADAB)-treated group which, despite small group sizes and very high mortality (all mice dying within 15 months), showed an increased risk for liver and lung tumours in both sexes and also for sarcomas in the males.

The risk analysis of the incidence of liver tumours showed a clear increase in risk associated with the increased rate of dieldrin exposure. The risk of lung-tumour occurrence was more difficult to assess. The analysis that assumed tumour responsibility for the death (i.e. non-incidental) indicated an increased risk, a result that was to some extent expected, as animals in the 10-ppm group were killed because of enlarged abdomens and were not allowed to die from natural causes. The second analysis, which assumed the tumour to be incidental, showed a slight increase in risk at the 0·1 and 1·0 ppm levels and a reduction at 10 ppm.

The problem is illustrated by considering the results for the analyses of lung tumours in males (Table 2). The values in Table 2 are the ratios between the number of tumours observed and the number expected for each treatment group, on the assumption that all mice, regardless of treatment, were exposed to the same risk. If the risk were equal, then all values should be close to unity; the test statistic, which is approximately distributed as χ^2 with four degrees of freedom, assesses the significance of the deviations from this expected value. A significant result indicates that there is a difference in risk between the treatment groups, and the ratios give an indication of the nature of that difference.

Both analyses show a significant difference between the treatment groups, with the highest risk attributable to ADAB despite the previously indicated high level of mortality. This example indicates an elevated risk of lung-tumour development for ADAB-treated mice. It is possible that excessive mortality would conceal an elevated risk of tumour development; this would not appear to be the case with ADAB and it is even more unlikely to apply to the group given 10 ppm dieldrin, since the mortality of the latter group was less than that of the ADAB-treated mice. No consistent increase in risk of lung-tumour development for dieldrin-treated mice is evident in the example or in other studies in which mice were exposed to dieldrin for periods up to 2 yr.

The conclusions with respect to tumour response in Carworth Farm No. 1 Strain mice to prolonged oral exposure to dieldrin, utilizing the techniques of relative risk analyses, are in agreement with the data reported earlier.

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

Dietary conen (ppm)	No. of animals	Liver: Type* (a)	Liver: Type* (b)	Liver: Total	Liver: With secondary tumour deposits in lung†	Percentage of animals with tumours of: Lung: Adenoma	Lung: Carcinoma	Lymphoid tissue	Kidney	Testes, Ovaries	Other tissues
Dieldrin											
Male											
0	289	16	4	20	0·7 (2)	32	7	35	9	1	7
0·1	124	22	5	27	0·8 (1)	38	11	22	11	2	3
1·0	111	23	9	32	0·9 (1)	37	13	22	6	—	5
10·0	176	37	58	95	0·6 (1)	18	1	3	1	—	2
Female											
0	297	12	0	12	—	17	6	41	1	15	7
0·1	120	18	3	21	—	19	10	37	1	8	8
1·0	117	23	5	28	0·9 (1)	25	10	44	—	11	12
10·0	148	37	59	96	4·1 (6)	11	—	5	—	1	1
ADAB											
Male											
600	23	13	4	17		39	—	9	—	—	61‡
Female											
600	21	43	48	90	9·5 (2)	29	5	5	—	—	—

*(a) Simple nodular growth of parenchymal cells; (b) areas of papilliform and adenoid growth of tumour cells.
†Figures in parenthesis are the actual number of mice showing this lesion.
‡Comprising haemangiosarcomas and anaplastic sarcomas.

Table 2. *Risk analysis of lung-tumour incidence in male mice*

Lung tumours in males	Ratio: tumours observed/tumours expected					
	Dieldrin (ppm)					
	Control	0·1	1·0	10·0	ADAB (600 ppm)	Test statistic
Non-incidental analysis	0·77	1·04	1·19	1·83	18·5	180***
Incidental analysis	0·94	1·16	1·14	0·72	2·07	17·6**

Asterisks indicate a significant difference in risk between treatment groups: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Fd Cosmet. Toxicol. Vol. 13, p. 601. Pergamon Press 1975. Printed in Great Britain

MEETING ANNOUNCEMENT

INTERNATIONAL CONFERENCE ON ENVIRONMENTAL MUTAGENS

The Second International Conference on Environmental Mutagens will be held in Edinburgh on 11–15 July 1977 under the patronage of H.R.H. The Duke of Edinburgh, K.G., K.T. Full details will be circulated to members of the American, European, Japanese and Indian Environmental Mutagen Societies. Other interested parties may obtain further information from the Secretary-General, Dr. P. Brookes, Institute of Cancer Research, Pollards Wood Research Station, Nightingales Lane, Chalfont St. Giles, Bucks HP8 4SP, England.

Fd Cosmet. Toxicol. Vol. 13, p. 602. Pergamon Press 1975. Printed in Great Britain

FORTHCOMING PAPERS

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

Studies on the metabolism of dimethylnitrosamine in the rat. II. The effects of phenobarbitone and 20-methylcholanthrene on the *in vitro* and *in vivo* metabolism and acute toxicity of dimethylnitrosamine in young and mature rats. By J. C. Phillips, Christine E. Heading, B. G. Lake, S. D. Gangolli and A. G. Lloyd.

Induction of neurogenic and lymphoid neoplasms by the feeding of threshold levels of methyl- and ethylnitrosourea precursors to adult rats. By A. Koestner, R. H. Denlinger and W. Wechsler.

Safety evaluation of yeast grown on hydrocarbons. IV. Two-year feeding and multigeneration study in rats with yeast grown on pure *n*-paraffins. By A. P. de Groot, Henriette C. Dreef-van der Meulen, H. P. Til and V. J. Feron.

Teratogenic evaluation of lead compounds in mice and rats. By G. L. Kennedy, D. W. Arnold and J. C. Calandra.

Observations on the oral administration and toxicity of vinyl chloride in rats. By V. J. Feron, A. J. Speek, Marianne I. Willems, D. van Battum and A. P. de Groot.

Biological testing of food grown in the Transkei. By I. F. H. Purchase, R. C. Tustin and S. J. van Rensburg.

A study of kinetic parameters for the use of serum ornithine carbamoyltransferase as an index of liver damage. By R. B. Drotman.

Dimethylnitrosamine in souse and similar jellied cured-meat products. By W. Fiddler, J. I. Feinberg, J. W. Pensabene, A. C. Williams and C. J. Dooley. (Short Paper)

The history and use of nitrate and nitrite in the curing of meat. By E. F. Binkerd and O. E. Kolari. (Review Paper)

Contents continued]

Aims and Scope

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

Instructions to Authors

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor. All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors (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:

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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.

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