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FOOD AND COSMETICS TOXICOLOGY

An International Journal published for the British Industrial Biological Research Association

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Research Section

Malformations Produced in Mice and Rats by Oxidized Linoleate

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(*Received 9 May 1973*)

Abstract—Linoleate oxidation products were investigated for teratogenic activity in rats and mice. Linoleic acid was oxidized at ambient temperatures until about 30% conjugated diene was present. The mixture was characterized and fed to the experimental animals at a level of 10% in the diet on 3 days/wk before and during pregnancy. Each treated rat received during pregnancy a total dose of 2·6–3·0 ml and each treated mouse a total dose of 1·2–1·6 ml. Malformations of the urogenital system occurred more frequently in the progeny of treated rats than in the offspring of control animals, but the linoleate oxidation products had no observable effect on the progeny of mice.

An investigation was then made of the embryotoxicity in mice of purified linoleic acid, oxidized linoleic acid or purified linoleic acid hydroperoxide applied directly to the ovaries. After the treatment with linoleic acid hydroperoxide an increase in foetal malformations occurred in litters of the first generation, and second-generation litters showed an increase in embryonic resorptions. After treatment with oxidized linoleic acid no change was detected in litters of the first generation but an increase in embryonic resorptions occurred in the progeny of the second generation. The incidence of malformations after treatment with linoleic acid was similar to that occurring in a group of untreated controls.

INTRODUCTION

In a previous communication (Cutler & Schneider, 1973) an increase in the incidence of tumours was reported among rats and mice that received oxidized linoleic acid in their diet.

Several compounds that increase tumour incidence in animals can also produce teratogenic and mutagenic effects (Hueper & Conway, 1964). Some of the products formed during autoxidation of polyunsaturated fatty acids are highly reactive and toxic compounds and these might interfere with cell division (Barber & Bernheim, 1967). For example, mutagenic effects can occur in Neurospora (Dickey, Cleland & Lotz, 1949) and in Aspergillus (Van Arkel, 1958) after exposure to fatty acid hydroperoxides. Fisher & Wilbur (1954) reported that irradiated fatty acids could initiate depolymerization of DNA in the rat.

In the experiments reported here, we investigated the possibility of teratogenic effects in rats and mice after the inclusion of oxidized linoleate in the diet. In addition, an examination was made of the effects on the progeny of mice after direct application of oxidized linoleic acid and purified linoleic acid hydroperoxide to the ovaries, and this was compared with the effects on the progeny after application of purified linoleic acid to the ovaries.

EXPERIMENTAL

Materials

Oxidized linoleic acid (OXLIN). Oxygen was bubbled through antioxidant-free linoleic acid for 72 hr at room temperature. The product was characterized by ultraviolet absorption

and by thin-layer chromatography on silical gel G plates using hexane–ether–acetic acid (60:40:1 by vol.) with iodine vapour as a visualizer. Identification of the spots was performed by the method of Freeman (1964). The mean composition of the batches of oxidized mixture is shown in Table 1.

Table 1. *Composition of OXLIN*

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

Values are means ± SEM.

Linoleic acid hydroperoxide (*LAHPO*). This was prepared at room temperature by direct aerial oxidation of a rotating film of antioxidant-free linoleic acid, which had been purified on a silica column. The hydroperoxide was separated from the oxidized mixture by partition between light petroleum and 75% aqueous methanol, as described by O'Brien & Frazer (1966). The purity of the isolated product, determined by ultraviolet absorption spectrophotometry, iodometry and thin-layer chromatography, was between 90 and 100%.

Linoleic acid (*LIN*). Antioxidant-free linoleic acid was obtained from Price's Ltd., Bromborough, Cheshire, and was purified on a silica column before use.

Examination of OXLIN for teratogenic activity

Animals. Rats from the Birmingham outbred Wistar strain and mice from the CBA strain were used in these studies. Both rats and mice had been reared by specific-pathogen-free foster parents and were kept in a barrier-controlled area of the animal house. Virgin females of both species were distributed by a random-number system between control and experimental groups, which contained about 20 animals, the exact number in each case being specified in the Results section.

Diets. The animals in the experimental groups received weighed amounts of a diet containing OXLIN for 3 days/wk from the age of 6 wk. Continuous feeding of the OXLIN was avoided in order to minimize toxic side-effects resulting from destruction of vitamins, essential fatty acids and protein in the diet (Kaunitz, 1953). A control diet of Spiller's rat cake was given *ad lib.* on the intervening days. The experimental diet was composed of 10% (v/w) OXLIN in rat-cake powder supplied by Spiller's Ltd., London, mixed for 30 min in a Hobart food mixer. This was freshly prepared each day. Control animals were given weighed amounts of Spiller's rat cake, to provide a food intake similar to the average food consumption of the experimental animals.

Experimental procedures. Female rats were mated at the age of 18 wk when weighing 250–300 g, and female mice at the age of 12 wk when weighing 20–25 g. Each female was

caged for 7 days with a male fed the basal diet. Males were then removed and females were caged individually until parturition. During pregnancy each rat in the experimental group consumed a total dose of 2·6–3·0 ml OXLIN and each experimental mouse a total dose of 1·2–1·6 ml OXLIN. All the animals born were weighed and sexed and critical autopsies were performed. Histological sections were prepared where necessary from tissues that appeared abnormal. The incidence of malformations among the progeny of animals given OXLIN was compared with that among the progeny of the control animals by means of the chi-squared test. Fisher's exact treatment of the 2 × 2 tables was also used where the numbers in the groups were small (Fisher, 1954).

Examination of the effects of OXLIN, LAHPO and LIN in the mouse-ovary painting test

This was performed by the method described by Kodikal & Batra (1965). Mice of the Birmingham outbred strain were anaesthetized with ether. The left and right sides were shaved and the surrounding fur was 'layed' with 1% cetrimide. The skin of the sides was cleaned with ethanol and the peritoneum was incised just above the ovaries and parallel to the ribs. A few spots of LAHPO, OXLIN or LIN were applied to the ovaries with a camel-hair brush. The incisions were stitched, the animals were caged singly and tetracycline was given in the drinking-water until the wound had healed.

The female mice were mated with normal males 14 days after the ovaries were painted. Of the 10–11 females in each group, 7 or 8 were killed within 24–48 hr of expected parturition, the foetuses and resorbed embryos being delivered by Caesarian section and autopsied under magnification. The chi-squared test and Fisher's exact treatment of the 2 × 2 tables were used to determine whether the incidence of malformations in experimental groups differed significantly from that observed in controls.

The remaining three females in each treatment group were allowed to give birth to their offspring and the offspring were brother–sister mated at the age of 6 wk. There were 7–10 pregnant F_1 females in each treatment group. All pregnant F_1 females were killed within 24–48 hr of expected parturition and the foetuses and resorbed embryos of the F_2 generation were examined in a similar manner to those of the F_1 generation. The significance of the difference between the groups in the incidence of malformations was estimated as before.

RESULTS

Teratogenic effects

As shown in Table 2, the offspring of rats given OXLIN in their diet showed a significantly higher incidence of malformations of the urogenital system than that observed among control animals. These malformations consisted of unilateral agenesis of a uterine horn and ovary, uterine hypoplasia, vaginal atresia, Walthard's inclusions of the ovary, pelvic kidneys and congenital cysts of the kidney. The incidence of other malformations in the litters of experimental rats was similar to that among the controls. The addition of OXLIN to the diet had no significant effect on fertility or on the sex ratio in the progeny.

As shown in Table 3, this effect on the urogenital system was not observed in mice. Addition of OXLIN to the diet of pregnant CBA mice was without any observable effect on the progeny.

Effects of local application of LIN, OXLIN and LAHPO to the ovaries of mice

Table 4 shows that there was a significant increase in the proportion of malformed foetuses among the first-generation progeny of mice of the LAHPO group and a corres-

Table 2. *Effects induced in the progeny of rats by the feeding of a diet containing 10% OXLIN before and during pregnancy*

Parameter	Values for groups fed	
	Control diet	OXLIN diet
No. of females mated	15	20
No. that conceived	12	16
Fertility: no. of pregnancies/no. mated (%)	80	80
Progeny		
Total no. born	134	182
Mean no./litter	11	11
Total no. dying without autopsy	3 (2)	9 (5)
Total no. with urogenital malformations	4 (3)	17 (9)*
Total no. with other malformations	5 (4)	7 (4)
Sex ratio (males/females)	0·82	0·68

Values in parentheses are expressed as percentages of the total no. of progeny, and that marked with an asterisk is significantly different from the control value: $*P < 0{\cdot}05$.

ponding decrease in the number of normal foetuses. Foetuses with developmental arrests were more frequent in the progeny of the LAHPO group than in the progeny of either the linoleate or untreated control groups, accounting for 8/61 (13%) of the conceptions in the LAHPO group compared with 3/54 (6%) in the linoleate control group and 3/72 (4%) in the untreated control group. This was significant at 95% confidence limits when the results from both control groups were combined. The other malformations were of several different morphological types, consisting of bifurcation of the ureters, adhesions of the adrenals to the kidneys with no intervening capsule, cleft palate, gross oedema and alterations in the numbers of digits and of ribs. The proportion of embryonic resorptions in the litters of the LAHPO group was similar to that observed among control mice.

Table 3. *Effects induced in the progeny of mice by the feeding of a diet containing 10% OXLIN before and during pregnancy*

Parameter	Values for groups fed	
	Control diet	OXLIN diet
No. of females mated	19	26
No. that conceived	15	24
Fertility: no. of pregnancies/no. mated (%)	79	92
Progeny		
Total no. born	97	153
Mean no./litter	6	6
Total no. dying without autopsy	12 (12)	23 (15)
Total no. with malformations	4 (4)	5 (3)
Sex ratio (males/females)	1·18	1·32

Values in parentheses are expressed as percentages of the total no. of progeny.

Table 4. *Effects induced in the progeny by local application of LIN, OXLIN or LAHPO to the ovaries of mice*

Parameter	Untreated control group	Values for mice treated with		
		LIN	OXLIN	LAHPO
	F_1 generation			
No. of females mated	8	7	7	8
No. that conceived	7	7	6	7
Fertility: no. of pregnancies/no. mated (%)	88	100	86	88
Total no. of fertilized ova	72	54	66	61
Total no. of resorbed embryos	15 (21)	7 (13)	11 (17)	12 (20)
Total no. of malformed foetuses	6 (8)	7 (13)	8 (12)	17 (28)**
Total no. of foetuses with developmental arrests	3 (4)	3 (6)	3 (5)	8 (13)*
Total no. of normal foetuses	51 (71)	40 (74)	47 (71)	32 (52)*
	F_2 generation			
No. of females mated	8	10	7	9
No. that conceived	8	9	7	9
Fertility: no. of pregnancies/no. mated (%)	100	90	100	100
Total no. of fertilized ova	80	92	56	92
Total no. of resorbed embryos	10 (13)	21 (23)	36 (64)**	57 (62)**
Total no. of malformed foetuses	8 (10)	9 (10)	2 (4)	6 (7)
Total no. of normal foetuses	62 (78)	62 (67)	18 (32)**	29 (32)**

Values in parentheses are expressed as percentages of the total no. of fertilized ova and those marked with asterisks are significantly different from the control group: *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

Application of either linoleic acid or OXLIN was without any significant effect on the progeny of the first generation. None of the substances applied to the ovaries had any significant effect on fertility.

Second-generation litters of both the LAHPO and OXLIN groups contained a significantly higher proportion of resorbed embryos than the second-generation litters of the linoleate group or untreated control group. The proportion of malformed foetuses in these groups was similar to that observed among controls. In the LAHPO and OXLIN groups, the proportion of resorbed embryos was significantly greater in litters of the second generation than in litters of the first generation.

DISCUSSION

There have been studies of chemical teratogenicity and mutagenicity in mammals for the past 30 years. These have demonstrated that teratogenic effects may be produced by several different mechanisms. Effects on the foetus may be the consequence of the direct toxicity of the compound under investigation or of its metabolites, but teratogenic effects can also result from indirect mechanisms, such as spasm of the umbilical cord and other changes produced in the foetoplacental unit, from nutritional changes in the mother or from antimetabolites or antivitamins (Sullivan, 1970).

In the present investigations, an increased incidence of urogenital malformations was observed in the progeny of rats fed diets containing oxidized linoleic acid. These malformations were of several types, including vaginal atresia, unilateral agenesis of the uterine horn and ovary, congenital cysts of the kidney and pelvic kidneys. Urogenital malformations of other types have been reported to occur in the offspring of rats deficient in folic acid or vitamin A (Auerbach & Barrow, 1972; Monie, Nelson & Evans, 1957; Wilson, Roth & Warkany, 1953). Other malformations have been observed in the progeny of rats deficient in pyridoxine (Davis, Nelson & Shepard, 1970) or in pantothenic acid (Roux & Dupuis, 1961). Since several dietary components, including vitamin A, biotin, pantothenic acid, pyridoxine, ascorbic acid, vitamin D and vitamin E, are said to be destroyed by oxidized fats and since the classical syndromes of vitamin A and vitamin E deficiency have been induced in animals by the feeding of heated fats (Lundberg, 1962), the malformations observed in the present experiments may merely reflect maternal vitamin deficiencies. However, the restriction of oxidized linoleic acid administration to only a few days of each week should have minimized such dietary deficiencies, and the only sign of vitamin deficiency observed in these animals was a decrease in the levels of serum and urinary folic acid (Cutler, 1969).

The results of the ovary painting test in mice suggest that changes in the hereditary material might have followed application of linoleic acid hydroperoxide to the ovaries. First-generation litters of these mice contained a higher proportion of malformed foetuses than that observed in either the linoleate or untreated control groups. Eight of these malformed foetuses showed developmental arrest, while the other malformations consisted of urogenital malformations, cleft palate, gross oedema and alterations in the numbers of digits and of ribs. Second-generation litters showed an increase in embryonic resorptions but the proportion of malformed foetuses was similar to that observed in the control groups.

After application of oxidized linoleic acid to the ovaries no change was detected in litters of the first generation, but an increase in embryonic resorptions occurred in the progeny of the second generation. The oxidized linoleic acid contained about 25% hydroperoxide (Table 1). The incidence of malformations after treatment with purified linoleic acid was similar to that occurring in the untreated control group.

Thus, direct embryotoxicity from linoleic acid hydroperoxide, which was one of the components of the oxidized linoleic acid used, may have played a part in producing the teratogenic effect observed in rats after inclusion of oxidized linoleic acid in their diet. The female rats each ingested about 650–750 mg linoleic acid hydroperoxide during pregnancy. However, maternal vitamin deficiencies might also have been involved in the teratogenic effect. Further studies to enlarge and confirm these findings would be of interest since the polyunsaturated fatty acids readily undergo autoxidation after exposure to free radicals and oxygen (Barber & Bernheim, 1967).

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Malformations provoquées chez la souris et chez le rat par le linolétae oxydé

Résumé—On a étudié sur des rats et des souris l'activité tératogène de produits de l'oxydation du linoléate. On avait oxydé l'acide linoléique à la température de la pièce jusqu'à ce qu'il contint 30% de diène conjugué. Le mélange standardisé a été distribué aux animaux d'essai à raison de 10% du régime, ceci 3 jours par semaine avant et pendant la période de gravidité. Pendant celle-ci, chaque rat traité a reçu une dose totale de 2,6 à 3,0 ml et chaque souris traitée une dose totale de 1,2 à 1,6 ml. Des malformations du système urogénital se sont produites plus fréquemment dans la descendance des rats traités que dans celle des animaux témoins, mais les produits de l'oxydation du linoléate n'ont pas eu d'effets observables sur la descendance des souris.

On a étudié ensuite l'embryotoxicité chez la souris de l'acide linoléique purifié, de l'acide linoléique oxydé et de l'hydroperoxyde d'acide linoléique purifié appliqués directement sur les ovaires. Après le traitement à l'hydroperoxyde d'acide linoléique purifié on a constaté une augmentation des malformations foetales dans les portées de la première génération et des résorptions d'embryons dans celles de la deuxième génération. Aucune modification n'a été décelée dans les portées de la première génération après le traitement à l'acide linoléique oxydé, mais des résorptions d'embryons se sont produites dans la descendance de la deuxième génération. La fréquence des malformations consécutives au traitement à l'acide linoléique était semblable à celle constatée dans un groupe d'animaux témoins non traités.

Missbildungen bei Mäusen und Ratten hervorgerufen durch oxydiertes Linoleat

Zusammenfassung—Linoleatoxydationsprodukte wurden auf teratogene Wirkung bei Ratten und Mäusen untersucht. Linoleonsäure wurde bei Raumtemperatur oxydiert, bis etwa 30% konjugiertes Dien vorhanden war. Die Mischung wurde charakterisiert und an die Versuchstiere mit einer Konzentration von 10% im Futter an drei Tagen je Woche vor und während der Trächtigkeit verfüttert. Jede Ratte erhielt während der Trächtigkeit eine Gesamtdosis

von 2,6-3,0 ml und jede Maus eine Gesamtdosis von 1,2–1,6 ml. Missbildungen des Urogenitalsystems traten häufiger bei den Nachkommen von behandelten Ratten auf als bei den Nachkommen von Kontrolltieren, jedoch hatten die Linoleatoxydationsprodukte keine beobachtbare Wirkung auf die Nachkommenschaft von Mäusen.

Dann wurde eine Untersuchung der Embryotoxizität von gereinigter Linoleonsäure, oxydierter Linoleonsäure oder gereinigtem Linoleonsäurehydroperoxyd, die direkt auf die Ovarien von Mäusen angewendet wurden, durchgeführt. Nach der Behandlung mit Linoleonsäure hydroperoxyd trat eine Zunahme der fetalen Missbildungen bei den Würfen der ersten Generation auf und Würfe der zweiten Generation zeigten eine Zunahme der Resorptionen. Nach der Behandlung mit oxydierter Linoleonsäure wurde keine Änderung bei Würfen der ersten Generation festgestellt, jedoch trat eine Zunahme der Resorptionen bei der Nachkommenschaft der zweiten Generation ein. Die Häufigkeit von Missbildungen nach der Behandlung mit Linoleonsäure war ähnlich der, die in einer Gruppe von Kontrolltieren auftrat.

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Dietary Feeding Studies of Methylcellulose and Hydroxypropylmethylcellulose in Rats and Dogs

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(*Received 12 June 1973*)

Abstract—Groups of ten male and ten female rats were fed for 90 days on diets containing methylcellulose (10 or 4000 cP) or hydroxypropylmethylcellulose (10 or 4000 cP) at concentrations of 0, 3 and 10% (and in some cases 1%). Groups of two male and two female beagle dogs received hydroxypropylmethylcellulose (10 cP) in their diets at concentrations of 0, 2 or 6% for 90 days. No evidence of toxicity was observed in either rats or dogs as judged by mortality, body weights, food-consumption values, urine analyses, haematological evaluations, levels of serum components, organ weights and gross and histopathological examination of tissues. Two long-term (2-yr) studies in rats were conducted on methylcellulose (15, 400 and 4000 cP) at dietary levels of 0, 1 and 5%, each dose level being fed to a total of 50 rats of each sex in the two studies. There was no evidence of treatment-related effects on mortality, body weights, food intake, haematology, levels of serum components, organ weights, gross and microscopic pathology or incidence of tumours.

These studies contribute additional evidence for the inert nature of the methylcellulose materials. Furthermore, the long-term studies indicate that methylcellulose does not possess tumorigenic properties.

INTRODUCTION

Methylcellulose, a polymeric carbohydrate derivative, has been an article of commerce in the USA since 1938. The hydroxypropyl modification of methylcellulose became available in 1948. Today, both of these types of cellulose derivative are used in a wide variety of food, drug and cosmetic products, in which they serve in a multifunctional way, imparting suspending, binding, stabilizing, film-forming, bulking, thickening, lubricating, emollient and protective-colloid properties, as required for specific end uses.

The very low oral toxicity of methylcellulose (Bargen, 1949; Bauer, 1945; Bauer & Lehman, 1951; Bauer, Lehman & Yonkman, 1944; Deichmann & Witherup, 1943; Ellingson & Massengale, 1952; *Journal of the American Medical Association*, 1945; Lehman, 1950; Machle, Heyroth & Witherup, 1944; Schweig, 1948; Tainter, 1943) and hydroxypropylmethylcellulose (Hodge, Maynard, Wilt, Blanchett & Hyatt, 1950; Knight, Hodge, Samsel, DeLap & McCollister, 1952; McCollister & Oyen, 1954; McCollister, Oyen & Greminger, 1961) has been well established.

Monographs have been prepared giving specifications for the identity and purity of methylcellulose (Joint FAO/WHO Expert Committee on Food Additives, 1964) and hydroxypropylmethylcellulose (*idem*, 1967a) and a toxicological evaluation (*idem*, 1964 & 1967a, b). On the basis of this FAO/WHO evaluation, an unconditional acceptable daily intake (ADI) of 30 mg/kg body weight has been established for methylcellulose and for hydroxypropylmethylcellulose, individually or collectively as the sum of the cellulose derivatives.

Specifications are also given for methylcellulose in the *United States Pharmacopeia* (1970) and for hydroxypropylmethylcellulose in the *National Formulary* (1970), and monographs on both of these methylcellulose products are included in the *Food Chemicals Codex* (1972)

Further confirmation of the very low oral toxicity of the methylcellulosics was provided by a statement from the National Research Council's Food Protection Committee (1970):

> "There are a number of substances, practically devoid of toxicity when taken orally, that are known to be not absorbed from the intestinal tract. Cellulose and a variety of artificially produced cellulose derivatives such as methylcellulose, sodium carboxymethylcellulose, and sodium cellulose sulfate, are examples. The cellulose derivatives can be fed at very high levels in the diet without toxic effects other than those related to reduction of nutrient intake or the induction of loose or bulky stools."

Methylcellulose is generally recognized as safe for its intended use (Title 21 of the Code of Federal Regulations, Sec. 121.101) and hydroxypropylmethylcellulose may be safely used in food according to a food additive regulation (21 CFR 121.1021). Both of these methylcellulose products are permitted as optional ingredients in French dressing (21 CFR 25.2) and salad dressing (21 CFR 25.3). In addition, there are numerous other food additive regulations in Title 21 of the Code of Federal Regulations covering a wide variety of uses for these products.

The studies summarized herein were conducted during the period from 1958 to the present, in order to maintain a continuing toxicological evaluation of methylcellulosics in current production.

EXPERIMENTAL

Materials. The methylcellulose products used in these studies are described in Table 1. The samples studied met the *United States Pharmacopeia* (1970) specification for methylcellulose and the *National Formulary* (1970) specification for hydroxypropylmethylcellulose. These products also met the specifications of the Food Chemicals Codex (1972) and the Joint FAO/WHO Expert Committee on Food Additives (1964, 1967a).

Animals and diets. Sprague–Dawley, Spartan-strain rats were used in the 90-day studies on methylcellulose 10 cP and 4000 cP and hydroxypropylmethylcellulose 4000 cP. Dow–Wistar rats were used in the 90–day study on hydroxypropylmethylcellulose 10 cP and in the 2-yr studies. Pure-bred beagles were obtained from a commercial breeder for the study in dogs. Rats were approximately 7 wk old and the dogs were about 7 months old at the beginning of each study. The rats were housed separately in wire-bottom cages in all studies except that on hydroxypropylmethylcellulose 10 cP, in which they were housed two in a cage. Pairs of dogs of the same sex and dietary level were housed together. The basal diet for both rats and dogs was ground Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.). Food and water were available *ad lib.*

Experimental design and conduct

Subacute studies in rats. Treatment groups in each study were composed of ten male and ten female rats randomized according to body weight. Dietary concentrations of 1, 3 and 10% were fed for 90 days in the studies on methylcellulose 10 cP and hydroxypropylmethylcellulose 10 cP. Methylcellulose 4000 cP and hydroxypropylmethylcellulose 4000 cP were

Table 1. *Samples used in dietary feeding studies on methylcellulose and hydroxypropylmethylcellulose*

Viscosity (cP)		Methoxyl groups (%)	Hydroxypropoxyl groups (%)	Loss on drying (%)	Residue (%) on ignition (sulphated)	Lot no.	Study
Nominal	Actual						
				Methylcellulose			
15	14·8	29·5	0	1·3	0·95	8052 & 7847	
400	375	30·0	0	0·6	0·21	8077 & 7775	2-yr/rat (2 studies)
4000	3740	30·3	0	1·1	0·21	8158	
10	10·0	28·8	0	0·6	0·81	MM010614C	90-day/rat
4000	4037	28·8	0	1·9	0·30	072022C	90-day/rat
				Hydroxypropylmethylcellulose			
10	11·1	29·0	8·2	1·4	1·56	301653X	90-day/rat and dog
4000	3588	28·2	6·2	1·4	0·65	242222F	90-day/rat

administered at concentrations of 3 and 10% in the diet, again for 90 days. Comparable control groups received the basal ration in each study.

Weekly body-weight and food-consumption records were kept. Haematological evaluations (packed cell volume (PCV) and haemoglobin (Hb) determinations, and erythrocyte (RBC) and total and differential leucocyte (WBC) counts) were conducted during wk 12 on five male and five female rats from the control groups and the groups fed the 10% diet of each material. Urine analyses (specific gravity or total solids, pH, glucose, albumin, ketones and occult blood) were conducted on five rats of each sex fed the 5 and 10% levels of methylcellulose 10 cP, methylcellulose 4000 cP and hydroxypropylmethylcellulose 4000 cP. Blood urea nitrogen (BUN) and serum alkaline phosphatase (AP) were determined terminally in all studies. Serum glutamic–pyruvic transaminase (SGPT) was also measured at the termination of studies on methylcellulose 4000 cP and hydroxypropylmethylcellulose 10 cP and 4000 cP.

Autopsies were performed on fasted rats after 90 days on test. Weights of brain, heart, liver, kidneys and testes were recorded and gross examinations were carried out on all rats. Histopathological examinations were conducted on haematoxylin–eosin-stained sections of selected tissues from rats fed the control and 10% diets. Most or all of the following tissues were examined in each study: thyroid, pituitary, trachea, lung, aorta, heart, liver, kidneys, adrenals, spleen, pancreas, stomach, small and large intestine, reproductive organs, urinary bladder, brain, spinal cord, peripheral nerve, skeletal muscle, bone marrow, mesenteric and mediastinal lymph nodes, and any lesions suggesting a possible pathological change.

Subacute study in dogs. Hydroxypropylmethylcellulose 10 cP was fed to two male and two female dogs at a concentration of 0, 2 or 6% in the diet. Body-weight and food-consumption data were recorded weekly. Values were obtained before the test and terminally for haematological parameters (PCV, Hb, RBC and total and differential WBC) and for BUN, AP, serum glutamic–oxalacetic transaminase (SGOT), SGPT and bromsulphthalein retention (BSP). Terminal autopsies were performed after 90 days on test, as described for the rat studies. Tissues from all dogs were examined histopathologically

Long-term study (I) in rats. Diets containing 1 or 5% methylcellulose 5, 400 or 4000 cP were fed to groups of 20 rats of each sex for 2 yr. Duplicate control groups, each of 20 males and 20 females, received the basal ration. Additional groups of ten rats of each sex for each treatment were set up for autopsy examination at 12 and 18 months. Body weights were recorded weekly throughout the study. Food consumption was measured during months 1 and 2. Rats dying during the study or sacrificed *in extremis* were subjected to gross pathological examination, and sections of lesions suggestive of tumour formation were preserved for microscopic evaluation. Haematological studies (PCV, Hb and total and differential WBC) were conducted at 12, 18 and 24 months on five male and five female rats from the groups fed the control ration and each of the 5% diets.

Each rat was examined grossly in autopsies conducted in rats maintained for 12, 18 and 24 months on test. The lungs, heart, liver, kidneys, spleen and testes were removed and weighed. Portions of these organs, along with the pancreas and adrenals, were preserved in formalin, and haematoxylin–eosin-stained sections were prepared for histopathological examination. BUN and AP were determined on serum obtained at autopsy.

Long-term study (II) in rats. In order to obtain data on tumour incidence from larger numbers of rats, diets identical to those fed in the first study as to sample and concentration were administered to groups of 30 male and 30 female rats for 2 yr. A control group of 30 rats of each sex received untreated diets. The rats were examined frequently for evidence of

tumour development. Body-weight records were maintained. Gross pathological examination was conducted on rats dying during the study or sacrificed *in extremis*, and sections of grossly visible nodules or masses were preserved for histopathological examination. Gross examination was conducted on all rats surviving at the end of 2 yr. Weights of liver and kidneys were recorded and grossly evident lesions suggestive of tumour formation were preserved for histopathological examination.

RESULTS

Subacute studies in rats

The rats appeared healthy throughout the studies. Mortality (Table 2) was random and was due to respiratory-tract infections unrelated to treatment. Soft stools and bulky faeces were observed in rats fed the 10% diets of each sample. Body-weight gains (Table 2) appeared to be slightly decreased in some of the groups of male rats receiving the higher dietary levels, although none of these values were significantly lower than those of controls (Student's *t* test). Food consumption was slightly but significantly increased in some of the treated groups (Table 2).

Haematological studies showed no treatment-related effects. Urinalyses gave similar results for treated and control rats. Levels of serum components (BUN, AP, SGPT) were all within the range of normal values.

Male rats receiving diets containing 10% methylcellulose 10 cP showed a significant decrease in their mean starved body weight at autopsy ($P < 0{\cdot}05$, Student's *t* test). The average weights in grams of a few of the organs from these rats were also significantly lower than controls. Final mean body weights of other groups of treated rats were not significantly different from corresponding controls. Other statistically significant increases or decreases occurred sporadically between organ weights of treated and control rats, but none of these were associated with treatment. Gross and histopathological examination of tissues revealed no lesions attributable to the ingestion of test samples. There was no microscopic evidence to indicate the accumulation of test material within the cells of the reticulo-endothelial system of treated rats.

Subacute study in dogs

The dogs were normal in appearance and demeanour throughout the study. Body-weight gain was comparable for treated and control dogs, with final weights ranging from 106 to 117% of initial weights. Average food consumption values for dogs fed the 0, 2 and 6% diets were 312, 340 and 324 g/dog/day, respectively, for males, and 235, 306 and 343 g/dog/day, respectively, for females.

Determinations of haematological elements, BSP retention and serum levels of BUN, AP, SGOT and SGPT made before the test and before termination gave similar values for treated and control dogs. Terminal organ weights and organ-to-body weight ratios revealed no differences indicative of a treatment-related effect. Gross and histopathological examination of the tissues revealed similar findings for control and treated dogs, with no evidence to indicate storage of the test material within the cells of the reticulo-endothelial system of the treated dogs.

Long-term study (I) in rats

All of the rats appeared normal and exhibited no unusual demeanour during the test period. Body weights were similar for rats receiving the 0, 1 or 5% diets (Table 3). Food-

Table 2. *Mortality. body-weight and food-consumption data for rats fed diets containing methylcellulose or hydroxypropylmethylcellulose for 90 days*

Sample	Nominal viscosity (cP)	Dietary concn (%)	No. of deaths	Body weight (g)		Food consumption (g/rat/day)
				Initial	Preterminal	
		Males				
Methylcellulose	10	0	0	252	493	28
		1	0	251	479	28
		3	0	250	479	28
		10	0	253	469	30***
		Females				
		0	0	201	305	22
		1	0	202	314	23
		3	0	200	311	21***
		10	0	198	300	23
		Males				
	4000	0	0	243	501	26
		3	0	247	521	29***
		10	0	243	509	30***
		Females				
		0	0	204	301	19
		3	0	203	304	20*
		10	1	203	292	21***
		Males				
Hydroxypropylmethylcellulose	10	0	0	240	344	23
		1	0	239	330	22
		3	0	237	317	22
		10	1	238	314	21
		Females				
		0	2	165	201	18
		1	2	165	199	18
		3	2	164	199	17
		10	1	164	206	17
		Males				
	4000	0	0	243	501	25
		3	0	245	516	29***
		10	0	246	492	32***
		Females				
		0	0	204	301	19
		3	0	204	302	21***
		10	0	204	296	23***

All groups initially contained ten rats.
Body weight and food consumption values are means for ten rats or the survivors thereof. Those marked with asterisks differ significantly (Student's t test) from those of controls: $*P<0{\cdot}05$; $***P<0{\cdot}001$.

consumption values likewise showed no compound-related differences. Mortality (Table 4) was unaffected by treatment and was associated with a variety of commonly observed spontaneous lesions. Haematological studies and determinations of BUN and AP gave normal findings, with no differences between control and treated rats indicative of a compound-

Table 3. *Body weights of rats fed diets containing methylcellulose for 2 yr (study I)*

Treatment		Body weight (g) at month								
Dietary concn (%)	Viscosity of methylcellulose (cP)	0	3	6	9	12	15	18	21	24
				Males						
0	(Control A)	139	313	336	376	382	394	395	393	395
	(Control B)	137	323	360	385	388	394	402	407	425
1	15	139	313	342	372	383	390	391	396	388
	400	140	314	350	383	396	403	403	413	403
	4000	139	327	360	388	396	404	410	402	415
5	15	140	306	358	384	384	404	412	416	405
	400	140	310	350	374	378	383	386	392	390
	4000	139	319	352	380	392	394	395	398	382
				Females						
0	(Control A)	117	197	220	238	250	266	275	278	276
	(Control B)	117	210	223	238	247	257	266	265	278
1	15	117	205	224	246	253	263	285	302	310
	400	117	194	212	230	242	250	263	268	268
	4000	117	210	232	247	261	270	286	296	292
5	15	119	204	225	238	248	257	258	267	266
	400	118	206	231	247	258	263	281	287	285
	4000	118	202	223	234	240	248	275	286	293

Values are the means of the body weights of all surviving rats in each group, composed of 20 rats at the start of the study.

related effect. Final mean body and organ weights of rats autopsied after 12, 18 and 24 months revealed a few random statistically significant differences, none of which was associated with treatment. Gross and histopathological examinations of the tissues revealed no compound-related changes, nor any evidence to indicate storage of the test material within the cells of the reticulo-endothelial system of treated rats. The tumours encountered were similar in type and number in treated and control groups (Table 5).

Table 4. *Mortality of rats in 2-yr feeding studies on methylcellulose*

Treatment		Mortality (%)			
		Long-term study I		Long-term study II	
Dietary concn (%)	Viscosity of methylcellulose (cP)	Males	Females	Males	Females
0	(Control A)	55	45	63	53
	(Control B)	50	45	—	—
1	15	55	50	57	43
	400	50	45	43	40
	4000	60	40	63	53
5	15	35	45	57	30
	400	50	55	57	40
	4000	55	50	47	47

Treatment groups in study I were each initially composed of 20 rats and those in study II of 30 rats.

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Table 5. *Tumours observed in rats fed diets containing methylcellulose for up to 2 yr (study I)*

Tumour location and classification	Viscosity of methylcellulose (cP)...	Tumour incidence in rats fed methylcellulose in a dietary concn (%) of						
		0	1			5		
		—	15	400	4000	15	400	4000
Lymphoreticular								
Malignant lymphoma		2	1	1	1	0	1	1
Subcutaneous								
Fibroma		0	0	0	0	1	1	1
Sarcoma		0	0	0	1	0	0	0
Mammary								
Fibroma		3	0	1	0	1	0	0
Sarcoma		0	0	0	0	1	0	0
Adenoma		0	0	1	1	0	0	0
Adenocarcinoma		0	1	0	0	0	0	1
Testes								
Interstitial cell		1	0	3	1	1	0	0
Adrenal								
Carcinoma		0	0	0	1	0	0	0
Kidney								
Lipoma		0	0	1	0	0	0	0
Heart								
Unclassified		1	0	0	0	0	0	0
Pleural cavity								
Unclassified		0	0	0	0	1	0	0
Uterus								
Fibroma		1	0	0	0	0	0	0
Carcinoma		0	1	0	1	0	0	0
Thymus								
Thymoma		1	0	1	0	0	0	0
Vagina								
Unclassified		0	0	0	0	1	0	0
Pancreas								
Carcinoma		0	0	1	0	0	0	0
	Total rats affected...	9	3	9	6	6	2	3

Treated groups were each composed of 20 males and 20 females, while the control group was composed of 40 males and 40 females.

Long-term study (II) in rats

The results of study I, summarized above, were duplicated in this study, mortality (Table 4), body weights and terminal liver and kidney weights being unaffected by ingestion of diets containing 1 or 5% methylcellulose 15, 400 and 4000 cP for 2 yr. The tumours observed are listed in Table 6. There was no indication of any increase in tumour incidence in rats receiving the treated diets.

DISCUSSION AND CONCLUSIONS

The methylcellulose products used in these studies are toxicologically similar to other methylcellulosics. The findings of loose and bulky stools, minimally decreased body-weight gain and slight increases in food intake recorded in the 90-day studies in rats have all been

Table 6. *Tumours observed in rats fed diets containing methylcellulose for up to 2 yr (study II)*

Tumour location and classification	Tumour incidence in rats fed methylcellulose in a dietary concn (%) of						
	0	1			5		
Viscosity of methylcellulose (cP)...	—	15	400	4000	15	400	4000
Lymphoreticular							
Malignant lymphoma	3	5	0	4	2	3	3
Subcutaneous							
Fibroma	1	0	0	1	1	0	1
Sarcoma	1	0	0	0	0	0	0
Fibrosarcoma	1	0	0	1	0	0	0
Mammary							
Fibroma	0	0	0	1	0	1	0
Adenoma	0	1	0	0	0	0	0
Fibroadenoma	0	1	0	0	0	0	0
Adenocarcinoma	1	1	1	0	0	0	0
Adrenal							
Pheochromocytoma	1	0	0	0	0	0	0
Carcinoma	0	0	0	0	1	0	1
Uterus							
Sarcoma	0	0	0	1	0	1	0
Fibrosarcoma	0	0	1	0	0	1	0
Adenocarcinoma	3	0	1	0	1	0	0
Squamous cell carcinoma	1	0	0	0	0	0	0
Carcinoma	0	0	0	0	1	0	1
Adenoma	0	0	0	0	2	0	0
Fibroadenoma	0	0	0	0	0	1	0
Unclassified	0	0	0	0	1	0	0
Thymus							
Thymoma	0	1	0	0	0	0	0
Vagina							
Fibrosarcoma	0	0	0	0	0	1	0
Liver							
Sarcoma	0	0	0	0	0	0	1
Lung							
Carcinoma	0	0	0	0	0	1	0
Intra-abdominal							
Mesenteric lipoma	0	0	1	0	0	0	0
Total rats affected...	12	9	4	8	9	9	7

Each group was composed of 30 males and 30 females.

reported in other investigations on methylcellulosics (Bauer & Lehman, 1951; McCollister & Oyen, 1954; McCollister *et al.* 1961).

The results of these studies contribute additional evidence of the inert nature of the methylcellulosics. Furthermore, the long-term studies indicate that methylcellulose does not possess tumorigenic properties.

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Etude nutritionnelle de la méthylcellulose et de l'hydroxypropylméthylcellulose chez le rat et le chien

Résumé—Des groupes de 10 rats mâles et de 10 rats femelles ont été soumis pendant 90 jours à des régimes à 0, 3 ou 10% (et dans certains cas à 1%) de méthylcellulose (10 ou 4000 cP) ou d'hydroxypropylméthylcellulose. Deux groupes de deux chiens bigles, les uns mâles et les autres femelles, ont consommé, également pendant 90 jours, de l'hydroxypropylméthylcellulose (10 cP) à raison de 0, 2 ou 6% du régime. A juger d'après la mortalité, le poids du corps, la consommation de nourriture, les analyses de l'urine, les évaluations hématologiques, les taux des composants du sérum, les poids des organes et l'examen général et histopathologique des tissus, aucun indice de toxicité ne s'est manifesté chez les rats ni chez les chiens. La méthylcellulose (15, 400 et 4000 cP) a aussi fait l'objet de deux essais de longue durée (2 ans) sur des rats. Les doses étaient de 0, 1 et 5% du régime et chaque dose a été

administrée à un total de 50 rats de chaque sexe dans chacun de ces essais. On n'a observé aucun effet imputable au traitement sur la mortalité, le poids du corps, la consommation de nourriture, l'hématologie, les taux de composants du sérum, les poids des organes, la pathologie générale et microscopique et les fréquences de tumeurs.

Ces essais apportent une preuve de plus de la nature inerte des méthylcelluloses et les essais de longue durée indiquent en outre que celles-ci sont dénuées de propriétés tumorigènes.

Verfütterungsversuche mit Methylcellulose und Hydroxypropylmethylcellulose an Ratten und Hunden

Zusammenfassung—Gruppen von zehn männlichen und zehn weiblichen Ratten erhielten 90 Tage lang Futter, das Methylcellulose (10 oder 4000 cP) oder Hydroxypropylmethylcellulose (10 oder 4000 cP) in Konzentrationen von 0,3 und 10% (und in manchen Fällen 1%) enthielt. Gruppen von zwei männlichen und zwei weiblichen Beaglehunden erhielten 90 Tage lang Hydroxypropylmethylcellulose (10 cP) in ihrem Futter in Konzentrationen von 0,2 oder 6%. Es wurde keine Anzeichen von Toxizität bei Ratten oder Hunden festgestellt, nach Mortalität, Körpergewicht, Futterverbrauchswerten, Urinanalyse, hämatologischen Untersuchungen, Konzentration von Serumbestandteilen, Organgewichten und makroskopischen und histopathologischen Untersuchungen der Gewebe zu urteilen. Zwei chronische (2 Jahre) Untersuchungen an Ratten wurden mit Methylcellulose (15, 400 und 4000 cP) mit Konzentrationen von 0, 1 und 5% im Futter durchgeführt, wobei jede Dosierung an insgesamt 50 Ratten jedes der beiden Geschlechter bei den beiden Untersuchungen verabreicht wurde. Es gab keine Anzeichen von mit dieser Behandlung im Zusammenhang stehenden Wirkung hinsichtlich Mortalität, Körpergewicht, Futterverbrauch, Hämatologie, Konzentrationen von Serumbestandteilen, Organgewichte, makroskopische und mikroskopische Pathologie und Häufigkeit von Tumoren.

Diese Untersuchungen liefern zusätzliche Informationen über den inerten Charakter der Methylcellulosematerialien. Ausserdem zeigen die chronischen Untersuchungen, dass Methylcellulose keine tumorerzeugenden Eigenschaften besitzt.

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Subacute Toxicity of Inorganic Tin as Influenced by Dietary Levels of Iron and Copper

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Abstract—The effects of various dietary levels of iron and copper on the changes induced by stannous chloride were examined in 4–6-wk feeding studies in rats. The severe growth retardation and distinct decrease in haemoglobin level caused by the addition of 5300 ppm tin to stock diet were diminished by dietary supplements of iron and/or copper. These effects occurred even though iron and copper were already present in the unsupplemented diet at levels several times higher than required. The decrease in the haemoglobin and serum-iron levels induced by adding 150 ppm tin to a semi-purified diet adequate in iron disappeared when the copper content was increased from 3 to 6 or 50 ppm. The growth depression and haematological changes caused by the addition of 500 ppm tin to a semi-purified diet just adequate in copper were less marked when the iron content was increased from 35 to 250 ppm. The addition of 150–1500 ppm tin to a semi-purified diet very high in copper and iron did not cause distinct haematological effects, but considerable growth depression remained.

These findings demonstrate that the feeding of diets containing high levels of tin results in anaemia unless high amounts of iron and copper are given. The growth depression caused by tin is not alleviated by enriching the diet with iron and copper. Obviously tin affects at least two different mechanisms, one related to haemoglobin synthesis and the other to growth.

INTRODUCTION

From previous short-term toxicity studies in rats, it appeared that the feeding of certain cationic tin compounds at levels of 0·3% or more in stock diet induced growth depression, signs of anaemia and histological changes in the liver, whereas no such effects occurred upon feeding the same levels of insoluble tin salts and oxides (de Groot, Feron & Til, 1973). Observations with diets of different composition indicated that the haematological effects produced by stannous chloride were diminished when the dietary level of iron was increased, whereas the effects were enhanced when the diet contained less iron (de Groot *et al.* 1973).

In order to obtain more information on the relationship between the toxicity of tin and the level of certain trace elements, further short-term experiments were carried out. The present report describes results obtained with stock diet and with semi-purified diets containing various levels of tin, iron and copper. Tin effects were assessed by determinations of gain in body weight, food intake and haematological criteria.

EXPERIMENTAL

Material. The tin compound used in these studies was stannous chloride 2 aq. (Analar, BDH Chemicals Ltd., Poole, Dorset). Ferrous sulphate 7 aq. and cupric sulphate 5 aq. (p.a., E. Merck AG, Darmstadt) were used as sources of iron and copper respectively.

Animals and diets. Male and female weanling rats of Wistar origin were housed in groups of five in stainless-steel screen-bottomed cages. Initial experiments were conducted with the Institute's stock diet (de Groot *et al.* 1973) which contains high and variable levels of iron (130–343 mg/kg) and copper (7–25 mg/kg). In subsequent experiments a semi-purified basal diet was used with low-iron casein as the source of protein (Zuid Nederlandse Melkindustrie N.V., Veghel). Its percentage composition was as follows: casein 20, sucrose 30·6, wheat starch 29, cellulose (Solkafloc) 5, vitamin ADEK-preparation 0·3, vitamin B mixture 0·3, choline preparation (50%) 0·4, *dl*-methionine 0·4, mineral mixture (Jones & Foster, 1942) 4, soya-bean oil 10. Diets low in iron or copper were obtained by omitting the salts of these metals from the mineral mixture. The residual iron and copper levels of these diets were found to be 7 and 3 ppm respectively.

Feeding studies. The diets containing various levels of tin, copper and iron were fed to groups of ten male and ten female weanling rats, or to ten males only. Food and drinking-water were offered *ad lib.* Test diets based on stock diet were offered in tin-plated cans, those based on the semi-purified diet in glass jars. Tap-water was used as drinking-water and was provided in glass bottles with a perforated rubber stopper and stainless-steel tube.

Body weights were recorded at weekly intervals for 4–6 wk. Haemoglobin measurements of the individual rats were made after 4 or 6 wk using blood obtained from the tip of the tail.

Determinations of serum iron and total iron-binding capacity were made by the method of Führ (1965) in pooled samples of decapitation blood from five or ten rats.

RESULTS

Experiments with stock diet

Mean values of body-weight gain and haemoglobin levels obtained in two successive experiments on stock diet supplemented with tin, iron and copper are shown in Table 1. The feeding of 1·0% stannous chloride (5300 ppm tin) resulted in considerable growth retardation and decreased haemoglobin levels. The supplementation of the tin diet with

Table 1. *Mean values for body-weight gain and haemoglobin levels of rats fed for 6 wk on stock diet supplemented with tin, iron and copper*

Dietary supplement(s)* (ppm)	Males		Females	
	Body-weight gain (g/rat)	Haemoglobin level (g/100 ml)	Body-weight gain (g/rat)	Haemoglobin level (g/100 ml)
	Experiment 1			
0	149	15·1	—	—
Tin (5300)	48***	12·3***	—	—
Tin (5300), iron (200), copper (75)	78***	14·3	—	—
	Experiment 2			
0	167	15·7	89	16·7
Tin (5300)	71***	12·8***	60***	13·6***
Tin (5300), iron (200)	78***	15·0	67***	15·5**
Tin (5300), copper (75)	76***	15·2	68***	15·5**

*1·0% $SnCl_2 \cdot 2H_2O$, 0·1% $FeSO_4 \cdot 7H_2O$ and/or 0·03% $CuSO_4 \cdot 5H_2O$.

Values are the means for groups of ten rats. Those marked with asterisks differ significantly (Wilcoxon's test) from the corresponding controls: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

iron (200 ppm) and copper (75 ppm) diminished slightly the growth depression caused by tin. The haemoglobin levels were raised considerably by the addition of iron and/or copper, but the values remained slightly lower than those of animals on the unsupplemented stock diet. The response to the separate addition of iron or copper was quantitatively very similar with respect to both gain in body weight and haemoglobin content.

It must be emphasized that the changes caused by supplements of iron and copper occurred in spite of the relatively high levels of these trace elements already present in the unsupplemented stock diet (220 and 18 ppm respectively).

Experiments with semi-purified diets

Tin effects at various dietary levels of copper. Results of an experiment with relatively little tin in the diet at three dietary levels of copper are presented in Table 2. The body weights of the rats were not affected by the feeding of 150 ppm tin in diets containing 6 or 50 ppm copper. Slightly decreased body weights were found, however, on the tin diet with only 3 ppm copper, although the difference was significant only in females.

Table 2. *Mean body-weight and haematological values of rats fed for 4 wk on semi-purified diets containing 56 ppm iron and supplemented with tin and/or copper*

Casein diet					
Copper content (ppm)	Tin content (ppm)	Body-weight gain (g/rat)	Haemoglobin level (g/100 ml)	Serum iron (μg/100 ml)	Total iron-binding capacity (μg/100 ml)
			Males		
50	0	120	14·2	165	596
50	150	119	13·9	165	586
6	0	119	13·8	175	553
6	150	119	13·3*	133	586
3	0	124	13·7	185	573
3	150	114	13·0*	128	590
			Females		
50	0	ND	14·3	ND	ND
50	150	ND	14·5	ND	ND
6	0	82	14·3	ND	ND
6	150	80	14·2	ND	ND
3	0	85	14·5	220	588
3	150	75·*	14·2	141	582

ND = Not determined

Values are the means for groups of ten rats. Those marked with asterisks differ significantly (Wilcoxon's test) from those of the corresponding controls receiving the same level of copper: $*P < 0{\cdot}05$.

The haemoglobin levels in the rats on the tin diets containing only 6 or 3 ppm copper were lower than in the corresponding controls, but in females the decrease was not significant. The differences became more marked with decreasing dietary levels of copper.

The levels of serum iron observed in rats on the tin diets were considerably decreased in both sexes with the diet containing the lowest level of copper. A similar effect, though less pronounced, occurred in males fed tin with the 6 ppm copper level. Females fed diets with 6 or 50 ppm copper were not examined for serum iron. The total iron-binding capacity of the blood was not noticeably affected by tin at any dietary level of copper.

Tin effects at high and low iron levels

Table 3 summarizes results obtained with three levels of tin in a semi-purified diet adequate in copper (6 ppm) and containing either a marginal (35 ppm) or a very high (250 ppm) level of iron.

Diets with 500 ppm tin caused growth depression, decreased food intake and food efficiency and decreased haemoglobin levels. These changes were more pronounced with the diet marginal in iron than with the high-iron diet. Rats fed the highest level of tin also showed lowered serum iron levels with diets both high and marginal in iron, while the total iron-binding capacity was slightly increased only with the diet marginal in iron.

With 150 ppm tin, gains in body weight, haemoglobin levels and serum-iron levels were consistently lower than on the corresponding control diets, but the differences were not more marked with the low iron diet. With 150 and 50 ppm tin, the food efficiency was not noticeably affected. The contents of serum iron were lower than in the controls but the differences were sometimes minimal and were not always correlated with the dietary levels of tin.

The results obtained with the 500 ppm tin diets, however, clearly indicated a more marked response when the iron content of the diet was reduced.

Tin effects at high levels of iron and copper

After observations had been made of the protective effects of copper and iron against the changes produced by tin, a final experiment was carried out with a semi-purified diet containing levels of iron and copper ten times higher than were required. From the results, shown in Table 4, it appears that a level of tin as high as 1500 ppm did not significantly decrease the haemoglobin levels, while the levels of serum iron were decreased only in females fed 500 and 1500 ppm tin. Growth and food efficiency, however, were considerably decreased at 1500 and 500 ppm tin in both sexes. Even at 150 ppm these values were lower than in the controls.

DISCUSSION

The results presented here show that certain changes induced in rats by dietary administration of stannous chloride are influenced by the level of iron and copper in the diet. The growth depression and the decrease in haemoglobin caused by the addition of a large amount of tin to stock diet were lessened by the addition of iron and copper, both singly and combined. This phenomenon occurred in spite of the very high levels of these trace elements already present in the basal diet. Six samples of stock diet taken at intervals during 2 yr were found to vary in iron content from 130 to 343 ppm (average 220) and in copper content from 7 to 25 ppm (average 18). These levels are considerably above the 35 ppm iron and 5 ppm copper required by the growing rat (National Academy of Sciences–National Research Council, 1972). It is clear, therefore, that neither the changes induced by tin nor the partial protection exerted by iron and copper can be attributed to a primary deficiency of the latter trace elements in the basal diet.

To enable the investigation of tin effects at lower and more constant levels of iron and copper, a semi-purified diet was used for further experiments. When this diet contained ample iron, the changes induced by 150 ppm tin were lessened by increasing the copper level from 3 to 6 or 50 ppm. A similar phenomenon occurred with a diet that contained 500 ppm tin and was just adequate in copper when the iron level was increased from 35 to 250 ppm.

Table 3. *Mean body-weight and haematological values of rats fed for* 4 *wk on semi-purified diets with* 6 *ppm copper supplemented with iron and/or tin*

Casein diet							
Iron content (ppm)	Tin content (ppm)	Weight gain (g/rat)	Total food intake (g/rat)	Food efficiency†	Haemoglobin level (g/100 ml)	Serum iron (μg/100 ml)	Total iron-binding capacity (μg/100 ml)
			Males				
250	0	125	298	0·42	13·9	222	643
250	50	127	295	0·43	13·9	195	639
250	150	119	285	0·42	13·1*	136	612
250	500	100*	259	0·39	12·3***	135	665
35	0	133	312	0·43	13·4	235	639
35	50	136	312	0·44	13·1	168	631
35	150	118	298	0·40	12·5*	201	669
35	500	90***	245	0·38	11·4***	169	761
			Females				
250	0	92	263	0·35	14·5	333	639
250	50	86	247	0·35	14·4	274	608
250	150	76*	239	0·32	14·2	166	596
250	500	74**	244	0·30	13·5**	176	578
35	0	85	253	0·34	14·1	259	586
35	50	89	274	0·32	14·7	253	594
35	150	80	243	0·33	14·0	243	551
35	500	65***	209	0·31	11·8**	116	686

†Food efficiency = weight gained (g)/food consumed (g).

Values are the means for groups of ten rats. Those marked with asterisks differ significantly (Wilcoxon's test) from the corresponding controls fed the same level of iron: *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Table 4. *Mean body-weight and haematological values of rats fed for* 4 *wk on semi-purified diets high in iron and copper* (250 *and* 50 *ppm respectively*) *and supplemented with tin*

Dietary tin supplement (ppm)	Weight gain (g/rat)	Total food intake (g/rat)	Food efficiency†	Haemoglobin level (g/100 ml)	Serum iron (μg/100 ml)	Total iron-binding capacity (μg/100 ml)
			Males			
0	130	305	0·43	15·4	183	588
150	121	298	0·41	15·3	243	590
500	94***	271	0·35	15·1	198	637
1500	52***	192	0·27	14·9	177	654
			Females			
0	85	251	0·34	16·2	335	548
150	75	239	0·31	16·2	348	571
500	69**	239	0·29	16·2	225	598
1500	41***	182	0·23	15·6	158	625

†Food efficiency = weight gained (g)/food consumed (g).

Values are the means for groups of ten rats, except for serum iron and total iron-binding capacity which were obtained on pooled blood samples of five rats. Values marked with asterisks differ significantly (Wilcoxon's test) from those of the controls: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Very high levels of iron and copper prevented haematological changes almost completely in animals fed semi-purified diets containing tin at levels up to 1500 ppm. However, growth depression and decreased food efficiency were noticeable first at 150 ppm and occurred to a considerable degree at 500 and 1500 ppm.

These findings demonstrate a markedly protective effect of iron and copper against the anaemia-inducing property of stannous chloride, whereas the growth-depressing effect of this tin compound was only slightly diminished by both trace minerals. This difference in response to iron and copper suggests that toxic tin levels act in at least two different ways, one affecting haemoglobin synthesis, probably by impairing absorption of iron and copper, and the other affecting growth rate and food efficiency.

In previous studies, 1500 ppm tin was the lowest level that induced distinct growth depression and decreased food efficiency in rats fed on stock diet for 4 or 13 wk (de Groot *et al.* 1973). In the present experiment with a semi-purified diet, these changes were noticeable initially at a tin level 10 times lower, although the amounts of iron and copper were comparable to those in the stock diet. This difference in results obtained with the two diets suggests that stock diet, in addition to its abundance in certain trace elements, possesses other properties which exert a protective action especially against the growth-depressing effect of tin.

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Toxicité subuiguë de l'étain inorganique selon les taux alimentaires de fer et de cuivre

Résumé—On a étudié par des essais nutritionnels de 4 à 6 semaines sur le rat les effets de différents taux alimentaires de fer et de cuivre sur les modifications causées par le chlorure stanneux. Des suppléments de fer et/ou de cuivre ajoutés au régime ont atténué l'important ralentissement de la croissance et la baisse marquée du taux d'hémoglobine provoqués par l'addition de 5300 ppm d'étain au régime de base. Ces effets se sont manifestés même quand le régime non supplémenté contenait déjà des taux de fer et de cuivre multiples des teneurs requises. La baisse des taux d'hémoglobine et de fer sérique provoquée par l'addition de 150 ppm d'étain à un régime semi-purifié à teneur en fer adéquate a cessé quand on portait la teneur en cuivre de 3 à 6 ou à 50 ppm. Le retard de croissance et les altérations hématologiques provoquées par l'addition de 500 ppm d'étain à un régime semi-purifié, dont la teneur en cuivre était juste adéquate, devenaient moins marqués quand on portait la teneur en fer de 35 à 250 ppm. L'addition de 150 à 1500 ppm d'étain à un régime semi-purifié très riche en cuivre et en fer n'a pas eu d'effets hématologiques notables, mais le grand retard de croissance a subsisté.

Ces constatations démontrent que les régimes à hautes teneurs d'étain provoquent l'anémie si l'on n'y ajoute pas de fortes doses de fer et de cuivre. Le retard de croissance imputable à

l'étain n'est pas atténué par l'enrichissement en fer et en cuivre du régime. Il est évident que l'étain influe sur au moins deux mécanismes différents, dont l'un intervient dans la synthèse de l'hémoglobine et l'autre dans la croissance.

Der Einfluss von Eisen und Kupfer im Futter auf die subakute Toxizität von anorganischen Zinn

Zusammenfassung—Die Wirkungen verschiedener Konzentrationen von Eisen und Kupfer im Futter auf die durch Zinn(II)-chlorid induzierten Veränderungen wurden mit 4- bis 6-wöchigen Verfütterungsversuchen an Ratten untersucht. Die starke Wachstumsverzögerung und die deutliche Abnahme der Hämoglobinkonzentration, die durch die Zugabe von 5300 ppm Zinn zum Normalfutter verursacht wurden, liessen sich durch Zusätze von Eisen und/oder Kupfer zum Futter abschwächen. Diese Wirkungen traten ein, obwohl Eisen und Kupfer schon vor dieser Ergänzung in mehrfach höheren Konzentrationen als erforderlich im Futter vorhanden waren. Die Abnahme der Hämoglobin- und Serumeisenkonzentrationen, die durch Zugabe von 150 ppm Zinn zu einem halbgereinigten Futter mit ausreichendem Eisengehalt herbeigeführt wurde, verschwand bei Erhöhung des Kupfergehalts von 3 auf 6 oder 50 ppm. Die Wachstumsverzögerung und die hämatologischen Veränderungen, die durch die Zugabe von 500 ppm Zinn zu einem halbgereinigten Futter mit gerade ausreichendem Kupfergehalt verursacht wurden, waren weniger deutlich nach der Erhöhung des Eisengehalts von 35 auf 250 ppm. Die Zugabe von 150–1500 ppm Zinn zu einem halbgereinigten Futter mit sehr hohem Kupfer- und Eisengehalt verursachte keine deutlichen hämatologischen Wirkungen, jedoch blieb es bei beträchtlicher Wachstumsverzögerung.

Diese Ergebnisse zeigen, dass Futter mit hohen Zinnkonzentrationen Anämie verursacht, wenn nicht grosse Mengen von Eisen und Kupfer gegeben werden. Die durch Zinn verursachte Wachstumsverzögerung wird durch Anreicherung des Futters mit Eisen und Kupfer nicht abgeschwächt. Offenbar beeinflusst Zinn mindestens zwei verschiedene Mechanismen, von denen einer mit der Hämoglobinsynthese und der andere mit dem Wachstum in Beziehung steht.

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Determination of Total Migration from Plastics-packaging Materials into Edible Fats Using a ^{14}C-labelled Fat Simulant

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Abstract—In the future, total migration may prove to be of great importance in the assessment of the physiological acceptability of new packaging materials, provided a practicable, reliable and sensitive method of determination is available. A suitable radio-tracer method has been developed using a ^{14}C-labelled standard triglyceride mixture, known as fat simulant HB 307-[^{14}C]. On the basis of the principle proposed by the Bureaux Internationaux Techniques des Matières Plastiques (BITMP), the sample of the packaging material is weighed before and after storage in fat simulant HB 307-[^{14}C]. The content of test fat remaining in and on the stored sample is determined by radio-analysis. The total migrate then equals the difference between (a) the weight of the sample before storage in the test fat and (b) the weight after storage less the weight of the fat absorbed by the sample during that storage.

This method was tested with specimens of plasticized PVC, LD-polyethylene, polystyrene and polyvinylidene chloride. The coefficient of variation of the single results about the mean showed a marked dependence on the amount of total migrate, the coefficients associated with 1, 6 and 12 mg total migrate/dm^2 surface of packaging material being 9, 2 and 0·6% respectively.

INTRODUCTION

Migration is the term used for the transfer of additives, monomers and other compounds under conditions of filling or storage (e.g. at 20°C and 65% relative humidity) from packaging materials into the products they contain or into simulants of such products. Transfer of a particular compound of toxicological interest is designated 'specific migration' whereas total or 'global' migration refers to the transfer of all mobile packaging components.

Unlike tests for specific migration, determination of the total migrate does not involve any direct toxicological considerations because both toxic substances and physiologically harmless packaging components are included. This test is therefore used at present only for checking new batches of already approved packaging materials, for which the total migration limits are known to the manufacturers. However, determining the total migrate from new packaging materials awaiting approval could give an important indication of total contamination and thus of the possible adulteration of the packed foodstuffs. Nevertheless, it has generally been considered that the determination of total migration is inadequate for a final physiological assessment of a new packaging material.

The Dutch Health Authorities have recently presented interesting proposals for the standardization and simplification of the analytical procedures for packaging-material approvals (Aldershoff, 1972), according to which the total migrate could serve as a basis for physiological assessment. It is suggested that approval of an additive for any type of packaging should be given only if the amount of the additive migrating from the final pack into the food contained in it does not exceed a maximum determined as acceptable

on the basis of animal trials. This means that the present limit on the amount of an additive in the packaging material would be replaced by a maximum allowable quantity of additive migrating into the foodstuff. A limit of 60 ppm (60 mg/kg food) is proposed for the tolerable total migrate in packaged foods.

Assuming a contact area of 5 dm^2/kg between the packaging material and the packaged food, the admissable total migrate is thus to be limited to 12 mg/dm^2 of the packaging surface. Exceptions to this rule are considered necessary in the case of plasticized PVC, paper, cellophane and rubber.

The German Federal Ministry of Health (Bundesgesundheitsamt) considers, however, that in view of the trend towards small packs, the total amount of migratable material should be fixed at a lower level of 6 mg/dm^2 packaging-material surface.

Methods are therefore needed for the accurate determination of 0·5 mg total migrate/dm^2 packaging-material surface. The use of aqueous simulants (e.g. 3% w/w acetic acid or 10% v/v ethanol) are unlikely to present difficulties, as these materials can be evaporated and the residues weighed accurately. Corrections can be made for possible errors due to the loss of volatile fractions of the migrate during evaporation of the simulant. In contrast, the total materials migrating into edible or test fats cannot be determined by this simple method.

Methods in which volatile solvents like *n*-heptane and diethyl ether are used as fat simulants and the total migrate is determined in the evaporation residue (Baumgartner, 1968; Federal Health Office, Berne, 1966; Italian Ministry of Health, 1966; US Code of Federal Regulations, Secs 121.2501 & 121.2514) are not appropriate for discussion here for reasons already outlined (Figge & Piater, 1972). The remaining methods are indirect, depending on the determination of the loss of migratable components from a packaging material in contact with a test fat. Of two approaches described in the literature, one is too inaccurate to be of value (BITMP (Bureaux Internationaux Techniques des Matières Plastiques), 1972; Brügger, 1971; de Wilde, 1966; Fluckiger & Rentsch, 1968; Robinson-Görnhardt, 1957 & 1958; Laboratoire cantonal de Berne, unpublished method, 1969). The second, more promising, approach involves weighing a packaging-material sample before and after storage in a test fat under defined test conditions. The difference between the two weights gives the total weight of the migrate, provided the test fat remaining in and on the packaging-material sample after storage is taken into account (BITMP, 1972). Methods based on this principle have the advantage that the same packaging-material sample is used in all steps of the investigation. However, the analytical methods currently used to determine the absorption of fat by the test film are relatively complicated and inaccurate (BITMP, 1971; Pfab, 1972; van Battum & Rijk, 1972; Wildbrett, Evers & Kiermeier, 1970).

Following a suggestion by Dr. C. G. vom Bruck of Unilever Forschungsgesellschaft mbH, Hamburg, we have now developed a generally applicable radio-tracer method, by which the total amount of material that has migrated into the fat simulant HB 307, a synthetic triglyceride mixture (Figge, 1972; Figge, Eder & Piater, 1972), can be estimated indirectly from the decrease in weight of the packaging material being tested. A representative sample of the packaging material is weighed before and after storage in the radioactively-labelled fat simulant (HB 307-[^{14}C]*) and the amount of HB 307-[^{14}C] retained

*The radioactivity in 1 kg HB 307-[^{14}C] is below the limit legally approved in the Federal Republic of Germany and most other countries.

Table 1. *Comparison of the amounts of additive migrating from different test films into edible fats and the fat simulant HB* 307 *during one-sided contact for* 60 *days at* 20°*C*

Test film*	Identity and concn (%, w/w) of labelled additive	Proportion (%) of radioactivity or additive migrating into				Ratio R† for		
		Biskin	Coconut oil	Butter	HB 307	Biskin	Coconut oil	Butter
Rigid PVC	Irgastab 17 MOK-[^{14}C]‡ (1·5)	0·009	0·014	0·017	0·016	1·8	1·1	1·0
HD polyethylene	Ionox 330-[^{14}C]§ (1·0)	0·090	0·098	0·120	0·140	1·6	1·4	1·2
	Stearic acid[1-^{14}C] amide (0·2)	0·80	0·96	1·05	1·36	1·7	1·4	1·3
Polystyrene	Ionox 330-[^{14}C] (2·0)	2·08	2·53	3·07	3·05	1·5	1·2	1·0
	n-Butyl stearate[1-^{14}C] (0·5)	5·20	5·61	7·11	7·57	1·5	1·4	1·1

*Figge *et al.* 1972.

†$R = \dfrac{\text{amount of additive migrating into fat simulant HB 307}}{\text{amount of additive migrating into Biskin, coconut oil or butter}}$.

‡Di-*n*-octyl[1-^{14}C]-tin-bis-(2-ethylhexylthioglycollate) (Figge, 1968).

§1,3,5-Trimethyl-2,4,6-tris-(3,5-di-*tert*-butyl-4-hydroxybenzyl[^{14}C])benzene (Figge, 1969).

in and on the sample in spite of careful cleaning is determined by radio-analysis. The suitability of the synthetic standard triglyceride mixture, HB 307, as a fat simulant is indicated by Table 1, which shows that the amounts of additives migrating from various test films into HB 307 are never more than 1·8 times as great as the values for the reference fats, Eiskin, coconut oil and butter. Moreover, additive migration from plastics into Biskin corresponds with that into margarine, olive and sunflower oils and other edible oils (Figge, 1971; Figge & Piater, 1971; Piater & Figge, 1971; vom Bruck, Figge, Piater & Wolf, 1971). The analytical advantages of using fat simulant HB 307 have been reported elsewhere (Koch, 1972).

EXPERIMENTAL

Principle of method for determining total migration into HB 307-[^{14}C]

The total migrate (GM) is calculated from the radio-analytically determined amount (mg) of fat absorbed (F_p) and the weight (mg) of the packaging-material sample before storage (Gv) and after storage (Gn) in HB 307-[^{14}C] (equation I):

$$GM = Gv - (Gn - Fp)\ (\text{mg}). \tag{I}$$

The advantages offered by this procedure are only realized if the determination of the fat absorption (Fp) by the test material is carried out rapidly and without interference and additional corrections.

HB 307 labelled with ^{14}C for use as a tracer substance in this context can reasonably be characterized only by its specific radioactivity, S_F, expressed in nCi or pCi/mg. Characterization by nCi/mmole is less accurate and is only possible if the mean molar mass of all the triglycerides in the fat simulant is known.

On the other hand, it must be recognized that triglycerides of different molecular size penetrate into plastics to differing degrees (Figge, 1971). It is therefore to be expected that from a triglyceride mixture like HB 307 the low-molecular triglycerides will migrate preferentially into the test materials, so that if the triglycerides of the fat simulant were labelled exclusively in their glycerol residues or only in the acyl residues, the specific radioactivity, S_F, of the original HB 307-[^{14}C], which is considered to serve as the operand, would not be identical with the specific radioactivity, S_{Fp}, of the simulant fraction, Fp, migrating into the test material. Consequently, during the determination of the fat absorption, Fp, an intentional systematic error would be introduced, differing from one packaging material to another. An accurate and rapid determination of the fat absorption, Fp, without the necessity of correction is possible by this radio-tracer method only when both the glycerol residue used for synthesizing the HB 307-[^{14}C] and each acyl residue possess radioactivity proportional to their respective molar masses.

It is a prerequisite that the radioactivity, A_{Fp} (nCi), migrating into the test material with the simulant fraction, Fp, is directly proportional to Fp:

$$A_{Fp} = S_{Fp} \cdot Fp\ (\text{nCi}). \tag{II}$$

On the other hand:

$$A_{Fp} = f_1S_1 + f_2S_2 + f_3S_3 + \ldots\ldots f_nS_n\ (\text{nCi}) \tag{III}$$

if f_i is the mass and S_i the specific radioactivity (nCi/mg) of the triglycerides with the same molecular weight present in the simulant fraction, Fp.

Equations II and III are then perfectly satisfied if:

$$S_{Fp} \cdot Fp = f_1 S_1 + f_2 S_2 + f_3 S_3 + \ldots\ldots f_n S_n \text{ (nCi)}. \quad \text{(IV)}$$

In practice, a rapid and accurate determination of Fp is only possible on the basis of equation IV if all S_i values are identical to each other and to the known specific radioactivity, S_F, of the original fat simulant HB 307-[^{14}C], in which case

$$\frac{A_{Fp}}{S_F} = \frac{A_{Fp}}{S_{Fp}} = Fp \text{ (mg)}. \quad \text{(V)}$$

As mentioned before, this aim is only reached if the glycerol residue and all the acyl residues contributing to the synthesis of HB 307-[^{14}C] possess the same specific radioactivity, s (nCi/mg), so that each triglyceride molecule in the simulant has a level of radioactivity, A_{Tr}, proportional to its molar mass:

$$A_{Tr} = s(m_{Gl} + m_{Ac(1)} + m_{Ac(2)} + m_{Ac(23)}) \quad \text{(VI)}$$

and $s = S_{Fp} = S_F$; m_{Gl} being the mass of the glycerol residue and $m_{Ac(1-3)}$ the individual masses of the three acyl residues in the triglyceride molecule.

Synthesis of HB 307-[^{14}C]

For the synthesis of the appropriate HB 307-[^{14}C], we were able to utilize the experience gained during the development of the inactive fat simulant HB 307 (Figge *et al.* 1972). We calculated the correct specific radioactivities of the reactants and esterified the mixture of synthetic [1-^{14}C]fatty acids with [1-^{14}C]glycerol without adding catalysts. The glyceride and fatty acid compositions of the purified radioactive reaction product corresponded within the limits of error with those of the inactive fat simulant HB 307.

Determination of fat in packaging materials

Errors in the determination of the fat content of packaging-material samples due to incomplete extraction of the fat from the test material can be excluded in most cases, and particularly in the case of plastics, by dissolving the samples in suitable solvents and using aliquots of these solutions for radioactivity measurements in a liquid scintillation counter. Test materials that are scarcely soluble or are insoluble are boiled immediately in a solvent with a good swelling effect or are thoroughly extracted. In order to control the course of the extraction, the increase in radioactivity in the extractant solution can easily be followed as a function of the extraction time. Clearly, the parallel tests for determining blank values and gravimetric determinations of the dry residues of extraction solutions required by the methods mentioned in the literature are not required in this case. Only the accurate determination of the specific radioactivity of the HB 307-[^{14}C] in nCi or pCi/mg is of significance.

Migration-test procedure with plastics films

From the packaging film a specimen 1 dm^2 in size is punched out using a square-puncher. Surface contamination is cleaned from the test film by careful rubbing with foamed plastics previously extracted three times with fresh supernatant ethanol at 60°C for 30 min. It is then kept for 24 hr at 20°C and 65% relative humidity and weighed accurately (Gv). For a further characterization of the test film, the mean thickness, D, can be determined. During this preparative work, the migration cell is packed with *c.* 120 g fat simulant, HB 307-[^{14}C],

which is equilibrated at 40°C. The cell consists of a glass vessel 13 cm high, 12 cm long and 1 cm wide. As illustrated in Fig. 1, the test film is fixed in a clamp and stored for 10 days in a bath of fat maintained at 40°C.

After storage, the test film is freed as far as possible from adhering test fat by careful dabbing with extracted foamed plastics at 40°C and is placed on foamed plastics. The specimen is then placed in the weighing sleeve, equilibrated for 24 hr at 20°C and 65% relative humidity and is weighed with care (*Gn*). First, the total weight of sleeve and film is determined, and then the sample is pushed into a 100-ml measuring flask with forceps and the empty sleeve is weighed alone.

The test film is then dissolved in the measuring flask, and the flask is filled up to the mark. Aliquots of this solution, generally five samples each of 2·0 ml, are measured in a suitable scintillation liquid using a liquid scintillation spectrometer. Since scintillation counting is a statistical measuring procedure, the measuring accuracy and detection limit depend mainly on the measuring time, i.e. long measuring times guarantee a high degree of accuracy. The radioactivity, A_{Fp}, of the fat-containing test film, in nCi or pCi, can be calculated directly from the mean of the individual measurements.

Scarcely soluble or insoluble test films are boiled in solvents with a marked swelling effect or are thoroughly extracted according to the known method in a Soxhlet apparatus. Aliquots of the concentrated solution of the extract are placed in a 100-ml measuring flask and made up to the mark. They are then measured as described previously.

The fat-simulant content (*Fp*) of the test film is calculated according to equation VII:

$$Fp = \frac{A_{Fp}}{S_F} \text{ (mg)} \qquad \text{(VII)}$$

the total radioactivity of the fat-containing test film (A_{Fp}, nCi) being divided by the specific radioactivity of fat simulant HB 307-[^{14}C] (S_F, nCi/mg).

All the data required for the calculation of the total migrate, *GM* are now available:

$$GM = \frac{Gv - (Gn - Fp)}{KF} \text{ (mg/dm}^2\text{)} \qquad \text{(VIII)}$$

where *Gv* is the weight (mg) of test film before and *Gn* the weight (mg) of test film after storage in HB 307-[^{14}C], *KF* is the contact area (dm^2) between test film and fat and *Fp* is the content (mg) of test fat in the stored test film. Equation VIII is free of correction terms since in the radio-tracer method the determination of blank values is unnecessary.

RESULTS AND DISCUSSION

Table 2 summarizes a series of results on various PVC films tested by this radio-tracer method. The investigations covered a large number of pressed PVC films containing 0–25% (w/w) of the plasticizer Palatinol AH (di-(2-ethyl-*n*-hexyl) phthalate). Some of the test films contained ^{14}C-labelled Palatinol AH, so that both the total migration and the specific transfer of Palatinol AH into the fat simulant HB 307 could be determined and related to the plasticizer content of the test film. Conditions were standardized, so the values obtained were directly comparable.

The total migration during all-sided contact and the specific migration of Palatinol AH from one-sided contact of the test films with the fat simulant HB 307 after a contact time of 10 days at 40°C appear in the right half of Table 2. From unplasticized PVC film, the

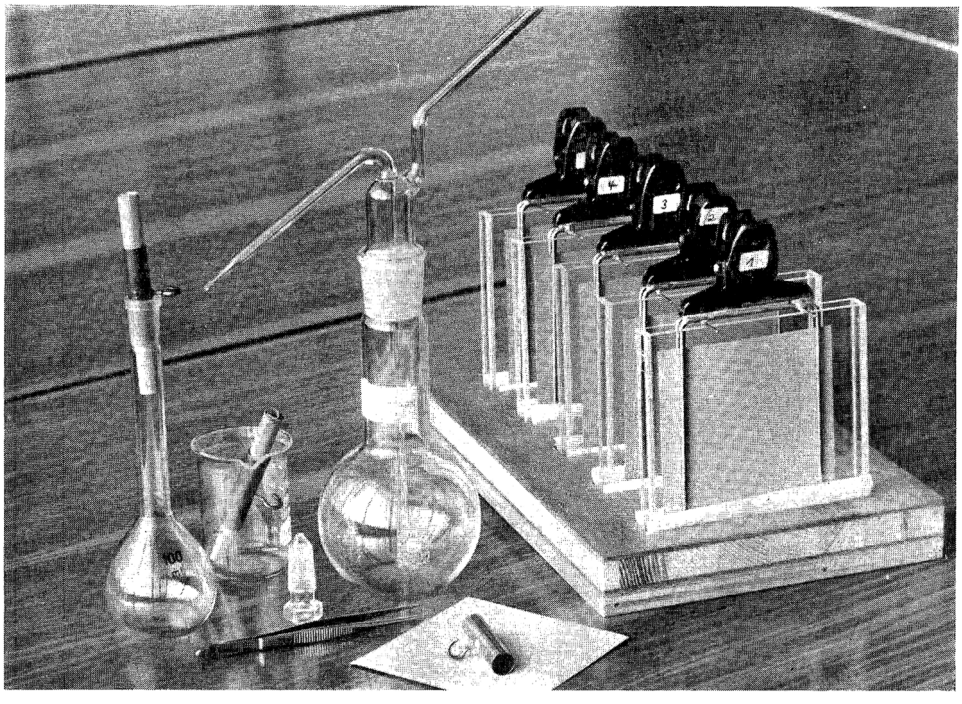

FIG. 1. Migration chambers and other apparatus for the determination of the total migrate.

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Table 2. *Specific migration of di-(2-ethyl-n-hexyl) phthalate (Palatinol AH) and total migration from PVC test films, plasticized to varying degrees, into fat simulant HB 307*

Characteristics of PVC test films*					Migration data after contact for 10 days at 40°C				
			Specific radioactivity of		Total migrate (*GM*) (two-sided contact)		Specific migrate (*SM*) (one-sided contact)		
Content of Palatinol AH (% w/w)	Thickness of film (μm)‡	Weight of film (mg/dm²)‡	Palatinol AH-[¹⁴C]† (μCi/g)‡	Test film (μCi/g)‡	mg/dm² film‡	% of film weight§	mg/dm² contact area	% of amount of additive in film	% of film weight§
No. of determinations (*n*)...	50	5	10	40	5				
0·00	235 (5·1)	3442·97 (2·7)	—	—	2·06 (8·7)	0·060	—	—	—
0·25	244 (1·8)	3447·86 (4·9)	—	—	2·76 (7·8)	0·080	—	—	—
0·50	246 (1·2)	3349·48 (0·5)	—	—	3·72 (7·0)	0·111	—	—	—
1·0	243 (4·1)	3257·91 (3·7)	—	—	5·94 (4·5)	0·182	—	—	—
4·0	222 (7·3)	2990·3 (3·0)	104·21 (1·17)	4·139 (2·71)	—	—	0·011	0·009	0·0004
5·0	234 (5·3)	3111·46 (3·7)	—	—	9·24 (2·6)	0·297	—	—	—
6·0	224 (11·2)	2994·6 (3·0)	70·92 (1·11)	4·225 (1·24)	—	—	0·078	0·043	0·0026
9·0	218 (11·8)	3003·2 (2·8)	34·94 (1·06)	3·162 (1·96)	—	—	1·324	0·58	0·044
10·0	226 (2·9)	2933·58 (1·6)	—	—	19·94 (0·8)	0·679	—	—	—
12·0	220 (7·0)	2960·3 (3·0)	34·94 (1·06)	4·155 (1·57)	—	—	7·21	2·04	0·244
15·0	215 (8·8)	2824·03 (8·4)	—	—	43·00 (0·40)	1·53	—	—	—
16·0	210 (5·5)	2758·5 (2·7)	34·94 (1·06)	5·823 (2·07)	—	—	38·26	8·30	1·39
20·0	212 (2·6)	2725·20 (1·6)	—	—	155·62 (0·34)	5·71	—	—	—
	216 (5·6)	2819·5 (2·8)	34·94 (1·06)	6·878 (2·24)	—	—	132·72	23·91	4·71
22·5	221 (4·5)	2684·96 (1·6)	—	—	296·68 (0·31)	11·05	—	—	—
25·0	205 (7·0)	2631·20 (5·9)	—	—	538·26 (0·32)	20·46	—	—	—
	213 (4·8)	2849·1 (2·7)	34·94 (1·06)	8·617 (1·58)	—	—	510·52	72·48	17·92

*Vinoflex 503 (supplied by BASF, Ludwigshafen). In addition to the plasticizer, the pressed films contained 0·5% (w/w) Wachs E (Farbwerke Hoechst, Frankfurt/M.) and 0·5% (w/w) stabilizer C (Farbenfabriken Bayer AG, Leverkusen).

†Di-(2-ethyl-*n*-hexyl) [7,8-¹⁴C]phthalate.

‡Values in parentheses are the coefficients of variation ($s\%$) of the single results about the mean of the no. of determinations indicated

$$(s\% = \frac{\text{standard deviation}}{\text{mean value}} \times 100).$$

§Calculated from *GM* in mg/dm² film or from *SM* in mg/dm² contact area and the film weight in mg/dm² film.

total migrate amounted to 2·06 mg/dm² film or 1·03 mg/dm² contact area. Increases of 5% in the plasticizer content of the film to provide concentrations of 5, 10, 15, 20 and 25% led to increases in the amounts of total migrate of 7·2, 10·7, 23·1, 112·6 and 382·6 mg/dm² film, respectively. The increase in total migration thus becomes larger the higher the plasticizer content of the film, as is also the case with the specific migration of Palatinol AH from PVC films into HB 307. This is clearly demonstrated in Fig. 2 in which the specific migration of Palatinol AH and the total migration into HB 307 are plotted as a function of the plasticizer content of the PVC test film.

In addition to the increasing steepness of the migration curves for PVC films containing more than 15% plasticizer, this graph also shows the close similarity between the values for total migration from two-sided contact and for specific migration of Palatinol AH from one-sided contact of the test film with the fat simulant over this range of plasticizer concentrations. This indicates that in a 10-day period at 40°C, a PVC film containing more than 15% Palatinol AH imparts approximately the same amount of plasticizer to HB 307 whether the contact is one-sided or two-sided. Consequently, the total migrate from a 1 dm² piece of a highly plasticized PVC film should not be halved to obtain the value for a contact area of 1 dm². It can in fact be seen from Fig. 2 that the curve obtained by halving the total migration values in this way and plotting them against the plasticizer content of the films does not conform to the actual migration as indicated by the curve of the specific migration of Palatinol AH. Halving of the total migration values is only appropriate if the test films are not swollen by the test fat, or are only swollen at the surface.

Five determinations of the total migrate were carried out for each test film, and from the individual results the mean total migrate and subsequently the coefficient of variation of the single results about the mean were calculated. In Fig. 3, this coefficient of variation of the single results has been plotted as a function of the mean total migrate determined with fat simulant HB 307-[^{14}C]. In the case of total migrates greater than 25 mg/dm² contact area or 50 mg/dm² film, this coefficient of variation amounts consistently to about 0·3%. In the range of the internationally discussed thresholds for total migrate of 6 and 12 mg/dm² packaging-material surface, a coefficient of variation of about 2 and 0·6%, respectively, must be taken into account. With a total migrate of 1 mg/dm² contact area, this error amounts to some 9%, but even this is a relatively small error if one considers the difficulties of weighing the test materials before and after storage in the test fat.

In addition to these experiments on PVC test films containing different concentrations of plasticizer, we determined the total migrates from two different low-density polyethylenes, from impact-resistant polystyrene and from polyvinylidene chloride into fat simulant HB 307-[^{14}C]. Table 3 lists the characteristics of the test materials and the migration results. In all cases the coefficient of variation of the single determinations about the mean was of the order of magnitude to be expected from the amount of total migrate.

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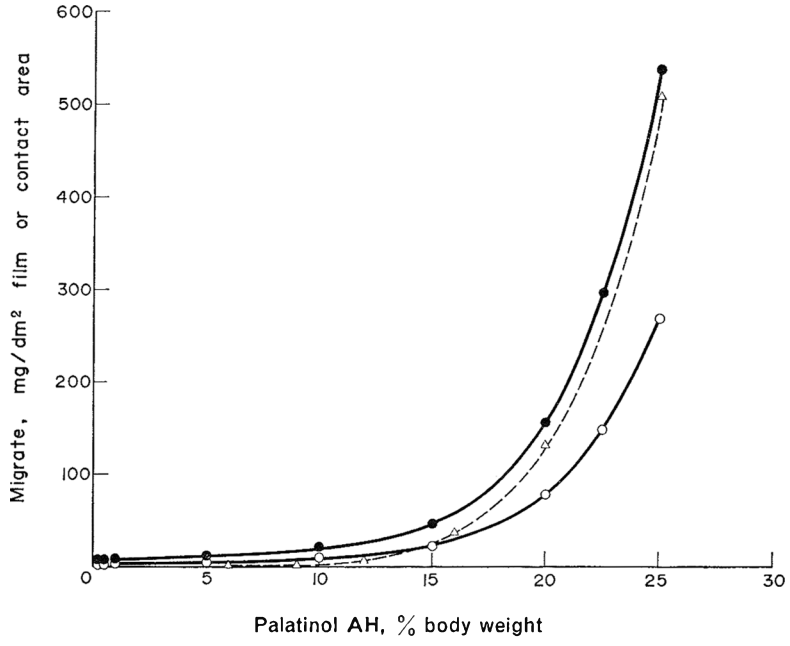

FIG. 2. Influence of plasticizer content of PVC test films on the specific migration (*SM*; △) of Palatinol AH (di-(2-ethyl-*n*-hexyl) phthalate) calculated on contact area and on total migration (*GM*) calculated on the size of film (●) and on the contact area (○), into the fat simulant HB 307-[^{14}C].

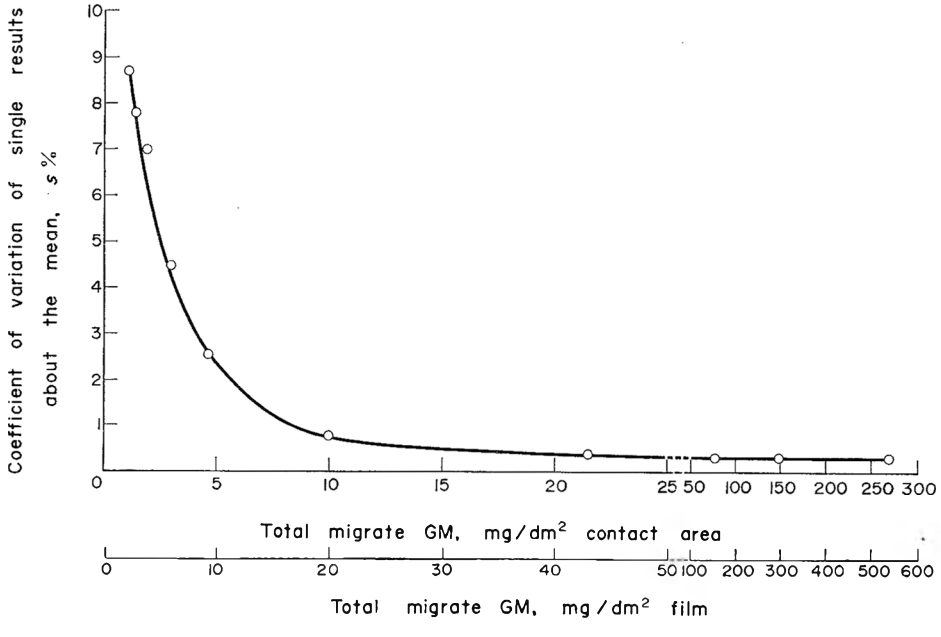

FIG. 3. Coefficient of variation of the single results about the mean ($s\%$) for the radio-tracer method as a function of the total migration (*GM*) determined with the fat simulant HB 307-[^{14}C]:

$$(s\% = \frac{\text{standard deviation}}{\text{mean value for 5 determinations}} \times 100).$$

Table 3. *Total migration (GM) from different plastics films into fat simulant HB* 307

Type of plastics*	Trade name	No. of determinations (n) ...	Characteristics of test films: Thickness of film (μm) 50	Characteristics of test films: Weight of film (mg/dm²) 5	Total migrate (GM) after 10 days at 40°C and two-sided contact: mg/dm² contact area 5	Total migrate (GM) after 10 days at 40°C and two-sided contact: % of weight of film†
LD-polyethylene	Lupolen 1810 H‡		103 (5·8)	1003·44 (6·3)	4·83 (3·21)	0·481
	Lupolen 1840 D‡		88 (15·4)	849·45 (14·7)	3·19 (3·95)	0·375
Impact-resistant polystyrene	476 L‡		104 (2·3)	1053·32 (2·3)	1·33 (10·53)	0·126
Polyvinylidene chloride§	—		104 (6·2)	1640·98 (3·6)	33·09 (0·51)	2·02

*The exact composition of the extruded films was not known.
†Calculated from GM in mg/dm² contact area and the film weight in mg/dm² film.
‡Supplied by BASF, Ludwigshafen.
§Containing 3% (w/w) dibutyl sebacate and 10% (w/w) Palamoll 646, a polyester of adipic acid and butane-1,3- and -1,4-diols supplied by BASF, Ludwigshafen.
Values in parentheses are the coefficients of variation (s%) of the single results about the mean of the no. of determinations indicated.

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Détermination à l'aide d'un modèle de matière grasse marqué au ^{14}C de la migration totale des plastiques d'emballage dans les graisses comestibles

Résumé—La migration totale pourrait acquérir à l'avenir une grande importance quand il s'agira de déterminer si des matériaux d'emballage nouveaux sont acceptables. Il faudra cependant disposer d'une méthode de détermination sensible, sûre et pratique. Les auteurs ont mis au point une méthode adéquate, à traceur radioactif, qui utilise un melange standard de triglycérides marqué au ^{14}C et appelé modèle de matière grasse HB 307-[^{14}C]. Conformément au principe proposé par les Bureaux Internationaux Techniques des Matières Plastiques (BITMP), on pèse l'échantillon de matériau d'emballage avant et après l'avoir déposé dans le modèle de matière grasse HB 307-[^{14}C]. La quantité de graisse de test qui reste dans et autour de l'échantillon ainsi traité se détermine par radio-analyse. Le "migrat" total est alors égal à la différence entre le poids de l'échantillon avant son séjour dans la graisse de test et le poids après ce séjour moins le poids de graisse absorbée par l'échantillon pendant le séjour.

Cette méthode a été essayée sur des spécimens de CPV plastifié, de polyéthylène basse densité, de polystyrène et de chlorure de polyvinylidène. Le coefficient de variation des résultats individuels par rapport à la moyenne dépend nettement de la quantité de "migrat" total, les coefficients relatifs à 1, 6 et 12 mg de "migrat" total par dm^2 de matériau d'emballage étant respectivement de 9, 2 et 0,6%.

Bestimmung der Migration von Zusätzen aus Kunststoff-Verpackungsmaterialien in Speisefette unter Verwendung eines ^{14}C-markierten Fettsimulans

Zusammenfassung—In der Zukunft könnte sich die Gesamtmigration als sehr bedeutsam für die Beurteilung der physiologischen Unbedenklichkeit neuer Verpackungsmaterialien erweisen, vorausgesetzt, es steht eine gut anwendbare, zuverlässige und empfindliche Bestimmungsmethode zur Verfügung. Eine geeignete radioaktive Markierungsmethode wurde unter Verwendung eines mit ^{14}C markierten Standard-Triglyceridgemisches, bekannt als Fettsimulans HB 307-[^{14}C], entwickelt. Nach dem von den Bureaux Internationaux Techniques des Matières Plastiques (BITMP) vorgeschlagenen Prinzip wird die Probe des Verpackungsmaterials vor und nach der Lagerung in dem Fettsimulans HB 307-[^{14}C] gewogen. Der Gehalt an Testfett, der in und auf der Probe verblieben ist, wird durch Radioanalyse bestimmt. Die Gesamtmigration ist dann gleich der Differenz zwischen (a) dem Gewicht der Probe vor der Lagerung in dem Testfett und (b) dem Gewicht nach der Lagerung abzüglich des Gewichts des Fettes, das von der Probe während dieser Lagerung absorbiert wurde.

Diese Methode wurde mit Proben von weichgemachtem PVC, Niederdruckpolyäthylen, Polystyrol und Polyvinylidenchlorid geprüft. Der Koeffizient der Schwankung der Einzelresultate um den Mittelwert zeigte eine deutliche Abhängigkeit von der Menge gewanderter Gesamtsubstanz, wobei die mit 1, 6 und 12 mg gewanderter Gesamtsubstanz/dm^2 Oberfläche des Verpackungsmaterials verbundenen Koeffizienten 9, 2 und 0,6% waren.

Fd Cosmet. Toxicol. Vol. 11, pp. 975–988. Pergamon Press 1973. Printed in Great Britain

Effect of Some Variables on the Migration of Additives from Plastics into Edible Fats*

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Abstract—It has been shown that organic liquids such as alkanes and di-*n*-alkyl ethers are unsatisfactory for use as fat simulants. A synthetic, analytically pure triglyceride mixture, which has a fatty acid and triglyceride distribution similar to that of coconut oil, has proved to be a universally applicable simulant for edible oils and fat-containing foodstuffs. The influence of temperature on the migration of additives from PVC, high-density polyethylene and polystyrene into edible oils and into this fat simulant, HB 307, has been studied. For all the polymer/additive combinations investigated, additive migration into the fat simulant increased markedly above a test temperature of 50°C. The intensity of this increase and the temperature at which it became apparent was found to depend on the type of plastics material and the physico-chemical properties of the additive. The amount of additive migrating is largely independent of the depth of the layer of HB 307 (i.e. the ratio of contact area to amount of simulant) as studies with simulant layers varying from 2·5 to 10·0 mm in depth showed. These results and others published elsewhere have been used to define conditions appropriate for the testing of plastics films and containers intended for packaging edible oils and fatty foodstuffs.

INTRODUCTION

When plastics are used for packing foodstuffs it must be remembered that these packaging materials consist not only of substances that are insoluble in foodstuffs but also of various auxiliary agents for facilitating the production, processing or use of the material. The types of plastics material and of foodstuff contained therein, as well as the intensity and duration of the contact between the container and the food, all influence the risk that these plastics additives could migrate into the food. Monomers and oligomers may also migrate into the food product.

In order to avoid the use of unsuitable packaging materials and consequently to protect the public from possible health hazards, national codes have been drawn up for the use of packaging materials by the food industry. To assess whether a packaging material conforms with these legal requirements, it is necessary to know the identity of the packaging material and its additives and the qualitative and quantitative transfer ('migration') of components of the packaging material into the foodstuff during the intended and foreseen use of the pack. To establish safety-in-use, the examination of the packaging material must be carried out on the material itself or on corresponding test specimens under the most severe conditions encountered in practice.

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For several years we have systematically investigated the migration of additives from plastics-packaging materials into fatty foodstuffs (Figge, 1971a,b & 1972a,b; Figge, Eder & Piater, 1972; Figge & Piater, 1971a–e & 1972; Figge & Schoene, 1970; Piater & Figge, 1971; vom Bruck, Figge, Piater & Wolf, 1971; vom Bruck, Figge & Wolf, 1970), and have developed a method for the determination of the migration.

Use of fat simulants in migration tests

The direct quantitative determination of additives migrating from plastics into edible oils or fatty foodstuffs (Woggon & Uhde, 1971) is extremely difficult even with the most sophisticated analytical techniques, and in some cases it may be impossible. Analytical procedures in which the migrated additives are concentrated or isolated from the fatty foodstuffs before their determination are tedious. Moreover they are inaccurate because of losses of material, and they may even fail completely. Therefore, in order to determine the migrated additives quantitatively in an analytically suitable simulant, it is essential to simulate the natural migration into foodstuffs in appropriate model tests.

Organic liquids such as alkanes, di-*n*-alkyl ethers and other solvents that have been suggested by various health authorities cannot be true fat simulants (Figge & Piater, 1971c & 1972; Piater & Figge, 1971), because their chemical structures differ completely from those of edible fats. Table 1 shows for different test films the ratios (R) between the quantities of additive migrating into alkanes or di-*n*-alkyl ether and into sunflower oil. Although in each case migration decreases with increasing chain length of the test liquid, migration of the named additives from films of HD-polyethylene and of polystyrene is considerably higher into all these organic solvents than into sunflower oil. Only the organotin stabilizer in the PVC test film migrates to approximately the same extent into alkanes, the di-*n*-octyl ether and sunflower oil. This conformity is probably due to the fact that the test film of rigid PVC is only swollen slightly or not at all by the sunflower oil and the named solvents, particularly the alkanes (Figge, 1971a). Consequently, the test solvent or oil dissolves only those quantities of additive that adhere to the film surface and can therefore be washed off (Figge & Piater, 1971b).

The high capacity of organic solvents for extracting plastics components compared with that of edible fats would be immaterial if the plastics additives were extracted in the same quantitative ratio by the test liquids and by edible fats, since it would then be possible to calculate the true migration rates with the aid of a few correlation factors. However, the alkanes and dialkyl ethers do not meet this requirement. For instance, whereas Ionox 330 and stearic acid amide migrate into both sunflower oil and tricaprylin in a ratio of 1:8, the ratio is 1:1 in the case of the migration into *n*-heptane and 1:6 with di-*n*-butyl ether. Moreover, the quantitative ratio of additives extracted from a plastics material alters with the chain length of the alkanes or dialkyl ethers. These and other experimental results (Figge & Piater, 1972) showed that organic solvents such as *n*-heptane, dialkyl ethers and others, are unsuitable for the simulation of fatty foodstuffs. Therefore, we developed a synthetic triglyceride mixture as a fat simulant (Figge *et al.* 1972). It can be seen from Table 2, that the amounts of additive migrating from different test films into this fat simulant, designated HB 307, exceed the values for reference fats by a maximum factor of 1·8. Moreover, we have already shown (Figge, 1971a; Figge & Piater, 1971b–e; Piater & Figge, 1971; vom Bruck *et al.* 1971) that the migration of additives from plastics into Biskin corresponds with that into margarine, olive oil, sunflower oil and other edible

Table 1. *Comparison of the amounts of additive migrating from different test films into alkanes, di-*n*-alkyl ethers or sunflower oil in* 60 *days at* 20°C *and* 65% *relative humidity*

Test film	Identity of labelled additive	*R** for Symmetric di-*n*-alkyl ethers† C_8	C_{10}	C_{12}	C_{16}	*n*-Alkane† C_7	C_9	C_{11}	C_{13}	Iso-octane
PVC	Irgastab 17 MOK-[^{14}C]‡	30	17	6·1	1·2	2·1	1·3	0·8	0·6	0·6
HD-polyethylene	Ionox 330-[^{14}C]§	23	15	11	6·0	55	40	31	21	16
	Stearic acid[1-^{14}C] amide	17	13	11	8·2	7·0	6·2	5·8	5·6	4·3
Polystyrene	Ionox 330-[^{14}C]	79	79	77	21	64	36	21	12	12
	n-Butyl stearate[1-^{14}C]	35	35	34	21	34	34	34	28	22

*$R = \frac{\text{amount of additive migrating into alkane or di-}n\text{-alkyl ether,}}{\text{amount of additive migration into sunflower oil}}$ both amounts being expressed as a percentage of the total amount in the test film.

†Total no. of carbon atoms/molecule.

‡Heat stabilizer, di-*n*-octyl[1-^{14}C]tin-bis-(2-ethylhexyl thioglycollate) (Figge, 1968).

§Antioxidant, 1,3,5-trimethyl-2,4,6-tris-(3,5-di-*tert*-butyl-4-hydroxybenzyl[^{14}C])benzene (Figge, 1969).

Table 2. *Comparison of the amounts of additive migrating from different test films into edible fats and the fat simulant HB* 307 *during one-sided contact for* 60 *days at* 20°*C*

Test film*	Identity and concn (%, w/w) of labelled additive	Proportion (%) of radioactivity or additive migrating into				Ratio R† for		
		Biskin	Coconut oil	Butter	HB 307	Biskin	Coconut oil	Butter
Rigid PVC	Irgastab 17 MOK-[^{14}C]‡ (1·5)	0·009	0·014	0·017	0·016	1·8	1·1	1·0
HD polyethylene	Ionox 330-[^{14}C]§ (1·0)	0·090	0·098	0·120	0·140	1·6	1·4	1·2
	Stearic acid[1-^{14}C] amide (0·2)	0·80	0·96	1·05	1·36	1·7	1·4	1·3
Polystyrene	Ionox 330-[^{14}C] (2·0)	2·08	2·53	3·07	3·05	1·5	1·2	1·0
	n-Butyl stearate[1-^{14}C] (0·5)	5·20	5·61	7·11	7·57	1·5	1·4	1·1

*For performance of migration tests and composition of test films see Figge & Piater (1971a) and Figge & Schoene (1970).

†$R = \dfrac{\text{amount of additive migrating into fat simulant HB 307}}{\text{amounts of additive migrating into Biskin, coconut oil or butter.}}$

‡Di-*n*-octyl[1-^{14}C]-tin-bis-(2-ethylhexylthioglycollate) (Figge, 1968).

§1,3,5-Trimethyl-2,4,6-tris-(3,5-di-*tert*-butyl-4-hydroxybenzyl[^{14}C])benzene (Figge, 1969).

oils. Thus, in contrast to the organic solvents, this synthetic standard triglyceride mixture represents a good and generally applicable simulant for pure edible fats and fatty foodstuffs.

Influence of temperature on migration rates

For edible fats and fatty foodstuffs packed in plastics materials, the normal storage time prior to sale and in the home is considered to be between 3 and 6 months at room temperature. Some years ago, van der Heide (1964) suggested an accelerated storage test for 10 days at 45°C for the rapid determination of migration. Using different systems of plastics and edible fats, he showed that under these proposed conditions the same additive transfer occurred as during storage at 25°C for 6 months. These findings were largely confirmed by Woggon and others (Woggon, Jehle & Uhde, 1969; Woggon, Uhde & Zydek, 1968).

In our earlier investigations (Figge & Piater, 1971d,e) we found that after 30 days at 20°C there is generally no longer a noticeable increase in the additive concentration either in edible fats or in fat simulant HB 307. Storage tests at 20°C, therefore, need not be continued for more than 30 days. Nevertheless, an accelerated test at increased temperature, such as a 10-day storage of the sample with HB 307 at 40°C, is still desirable.

By means of the method we have developed, we have investigated the influence of temperature on the migration of additives from a series of commercial plastics materials into edible fats or the fat simulant HB 307.

EXPERIMENTAL

Materials. The plastics compositions studied and the sources of the materials are summarized in Table 3. The polystyrene, HD-polyethylene and PVC compositions, which are all widely used for packaging fatty foods, were compounded with the ^{14}C-labelled additives listed in column 3 of Table 3. Thus, for both polystyrene and polyethylene, two compositions were prepared differing only in the additive that carried the radioactive labelling. All these mixtures were extruded to test films by means of a laboratory extruder. The physico-chemical properties of the films were comparable with those made on a factory scale. It was confirmed that the radio-indicators were evenly distributed throughout the test films (Figge & Schoene, 1970).

Test procedures. The HD-polyethylene and PVC films were kept in one-sided contact with the fat simulant HB 307 for up to 30 days at the required temperatures. The practical basis of these tests is discussed elsewhere (Figge, 1972a; Figge & Piater, 1971a). Since polystyrene films have a tendency to exhibit stress cracking or to soften at raised temperatures, they could not be clamped in the migration cells and were therefore cut into chips and maintained in all-round contact with HB 307 under otherwise comparable conditions.

Influence of temperature and time on migration. For all the test films, the amounts of labelled additive migrating into HB 307 were determined at a range of storage temperatures between 30 and 80°C. The time course of the migrations was also studied between 1 and 30 days.

Influence of depth of HB 307 layer on migration. To determine the extent to which the ratio between the contact area and the volume of simulant (i.e. the depth of the layer of HB 307 on the test material) affects the amount of migration, migration cells of differing width but otherwise identical construction were designed. The basic design of these cells has been described by Figge & Piater (1971a) and is shown in Fig. 1. With a constant area of contact, the layers of simulant between pairs of test films fixed in the cells parallel to

Table 3. *Characteristics of test films used for migration studies*

	Test films								
		^{14}C-labelled additive							
Plastics mixture	Designation	Identity	Specific radioactivity (μCi/g)	Specific radioactivity μCi/g film	s%* (n)	Thickness μm	s%* (n)	Weight mg/dm^2	s%* (n)
Polystyrene†	PS-Io	Ionox 330-[^{14}C]	109·28	2·0311	0·45 (30)	379	8·5 (30)	3271	2·69 (10)
	PS-Sb	*n*-Butyl stearate[1-^{14}C]	415·88	1·8360	1·35 (30)	243	7·0 (30)	2283	7·44 (10)
HD-polyethylene‡	HD-PE-Io	Ionox 330-[^{14}C]	219·07	2·1244	1·85 (30)	345	12·0 (320)	3350	5·71 (40)
	HD-PE-Sa	Stearic acid[1-^{14}C] amide	1192·56	2·1613	1·83 (30)	306	13·0 (480)	2870	13·03 (60)
PVC§	PVC-Sn	Irgastab 17 MOK-[^{14}C]	684·13	9·1956	1·50 (20)	329	1·96 (168)	4411	0·72 (21)

*$s\% = \frac{\text{standard deviation}}{\text{mean value of } n \text{ single values}} \times 100$, the number of single measurements (n) being given in brackets.

†Composition: 97·5% (w/w) KR 2570/2 (BASF, Ludwigshafen), 2·0% Ionox 330 (1,3,5-trimethyl-2,4,6-tris-(3,5-di-*tert*-butyl-4 hydroxybenzyl)benzene; Shell Chemical Corp., USA), 0·5% *n*-butyl stearate.

‡Composition: 98·8% (w/w) Lupolen 5261-Z (BASF, Ludwigshafen), 1·0% Ionox 330 and 0·2% stearic acid amide.

§Composition: 97·0% (w/w) Solvic 229 (Deutsche Solvay Werke, Düsseldorf), 1·5% Irgastab 17 MOK (di-*n*-octyltin-bis-(2-ethylhexyl thioglycollate); Ciba Geigy Marienberg GmbH, Marienberg), 0·5% Ionox 330, 0·6% Stenol 1618 (cetylstearyl alcohol; Deutsche Hydrierwerke GmbH, Düsseldorf), 0·4% Loxiol G10 (ricinoleic acid monoglyceride; O. Neynaber, Loxstedt).

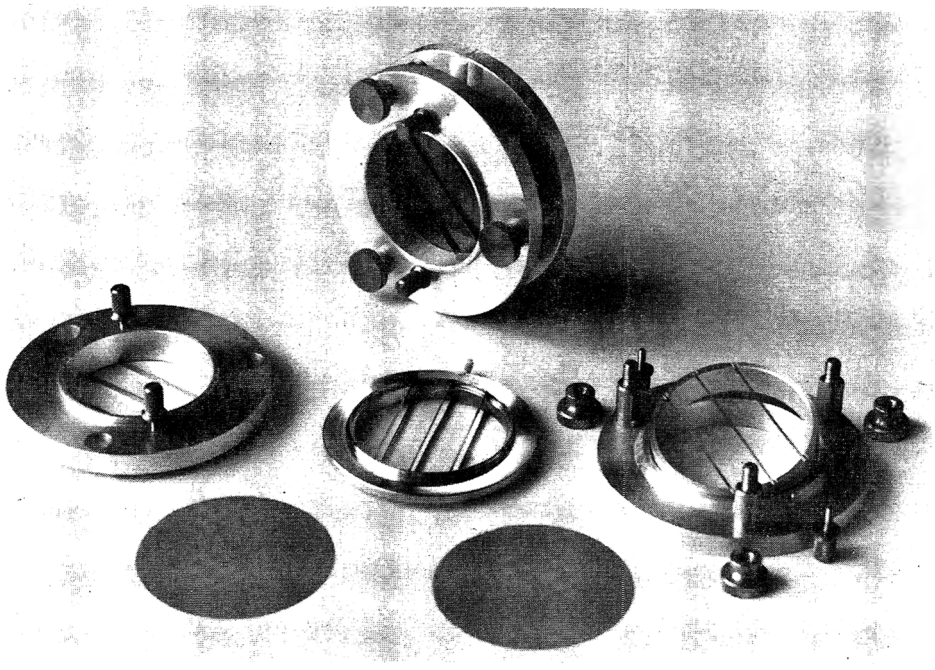

FIG. 1. Migration cell

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each other were exactly 5, 10 or 20 mm thick. These studies were carried out with pressed films of PVC containing levels of Palatinol AH-[^{14}C] between 4 and 25 % (w/w) and extruded HD-polyethylene film containing 1 % (w/w) of the labelled antioxidant Ionox 330-[^{14}C]. For each test film, five separate determinations of plasticizer migration were carried out with each depth of simulant studied and the mean amount of plasticizer was calculated in each case from the five determinations. In addition, for each test film, the mean amounts of transferred plasticizer and the coefficient of variation of the single results about the mean were calculated from all 15 separate determinations, thus giving a mean migration value independent of the depth of the simulant layer.

RESULTS AND DISCUSSION

The results of these studies were used to determine the temperature, contact time and fat-simulant volume most suitable for the execution of these migration tests. Considering, for instance, the migration of *n*-butyl stearate from the polystyrene film into HB 307 (Fig. 2),

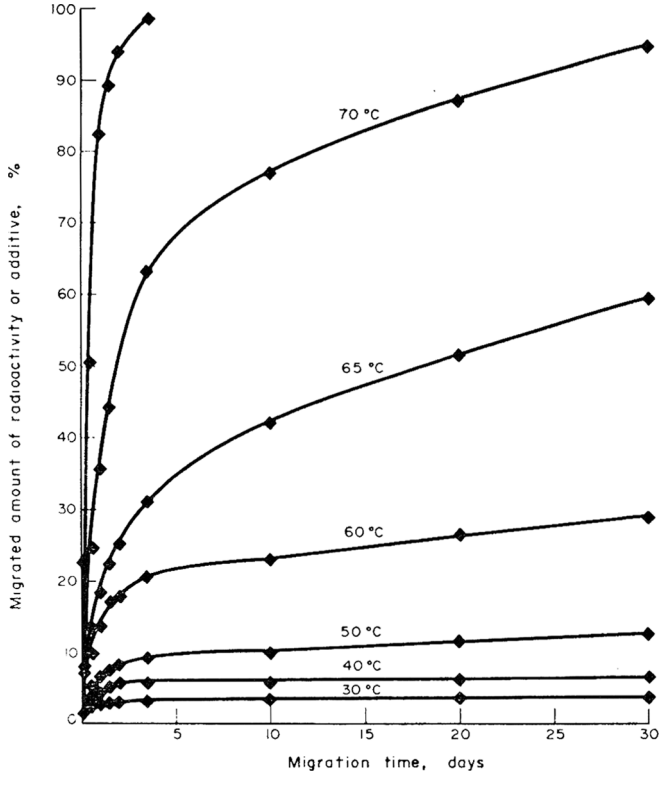

FIG. 2. Migration of *n*-butyl stearate[1-^{14}C] from polystyrene film into fat simulant HB 307 during periods of 1–30 days at temperatures between 30 and 80°C.

it can be seen that at 40°C, in contrast to the situation at higher temperatures, there was no further migration of the additive after 10 days. In fact, in this case, the final migration value, amounting to 5·68 % of the amount of additive in the original film, was reached after only 4 days.

Figure 3 shows the amounts of additives migrating in 10 days from the test films into HB 307 at various test temperatures. With all the polymer/additive combinations studied, there was a marked increase in migration values above 50°C. The extent of this increase and the point at which it began depended both on the type of plastics material and the physico-chemical properties of the additive. Thus, while the migration of Ionox 330 from either polystyrene or HD-polyethylene showed a marked increase only above 65°C, an increase in the migration of *n*-butyl stearate from polystyrene and of stearic acid amide from HD-polyethylene was clearly observed at 50°C. Consequently, in order to simulate migration under storage conditions, it is necessary to know the extent of this increased migration and the temperature at which it begins to occur, since the use of too high a test temperature could indicate an unrealistically high level of additive transfer.

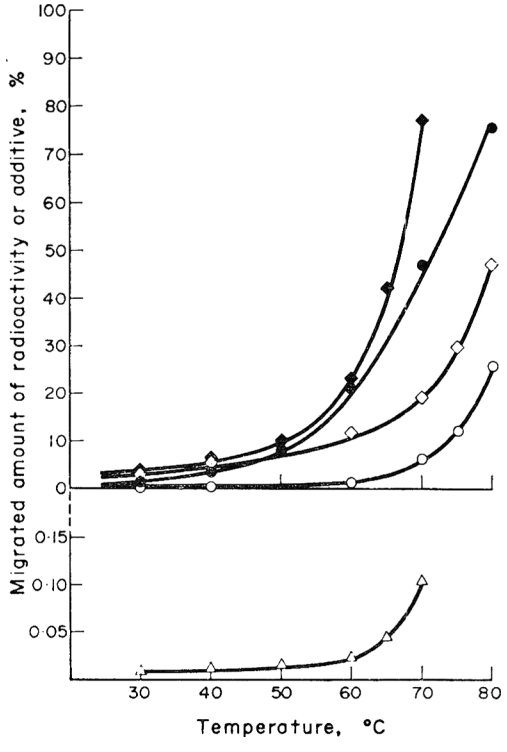

FIG. 3. Migration of labelled additives (Ionox 330 (○———○) and stearic acid amide (●———●) from HD-polyethylene film, Ionox 330 (◇———◇) and *n*-butyl stearate (◆———◆) from polystyrene film and Irgastab 17 MOK (△———△) from PVC film) into fat simulant HB 307 during a 10-day period at temperatures between 30 and 80°C.

Comparison of the amounts of additives that had migrated from the test films into HB 307 after 30 days at 20°C and after 10 days at 40°C (Table 4) indicates that the migration of additives from plastics packs into fatty foods stored at about 20°C can be simulated satisfactorily in tests with HB 307 for 10 days at 40°C.

Table 4. *Comparison of the amounts of additive migrating from different test films into fat simulant HB* 307 *and coconut oil*

Test film	Labelled additive	Migration of additive (%)		
		After 30 days at 20°C into		After 10 days at 40°C into HB 307
		Coconut oil	HB 307	
PVC	Irgastab 17 MOK-[^{14}C]	0·010	0·014	0·012
HD-polyethylene	Ionox 330-[^{14}C]	0·080	0·120	0·106
	Stearic acid[1-^{14}C] amide	3·86	3·48	3·66
Polystyrene	Ionox 330-[^{14}C]	4·89	4·76	5·58
	n-Butyl stearate[1-^{14}C]	5·27	5·42	5·76

Figure 4 demonstrates that these facts also apply to rigid PVC films, which, as already mentioned, are not swollen by fats at temperatures up to 60°C. Two different rigid PVC films (Figge & Zeman, 1973) containing the same stabilizer, Irgastab 17 MOK labelled with ^{14}C in the octyltin group were each found to impart the same amounts of additive to HB 307 within 30 days at 20°C as within 10 days at 40°C. The amounts of radioactivity or stabilizer migrating from the test film into the fat simulant were approximately equal under the different test conditions and with both films the migration reached a constant final value before test periods expired.

Details of the study on the migration of plasticizer from PVC films into different depths of HB 307 layers are given in Table 5. The extent of migration of Palatinol AH in 10 days at 40°C increased markedly with increasing plasticizer content and the concentration of

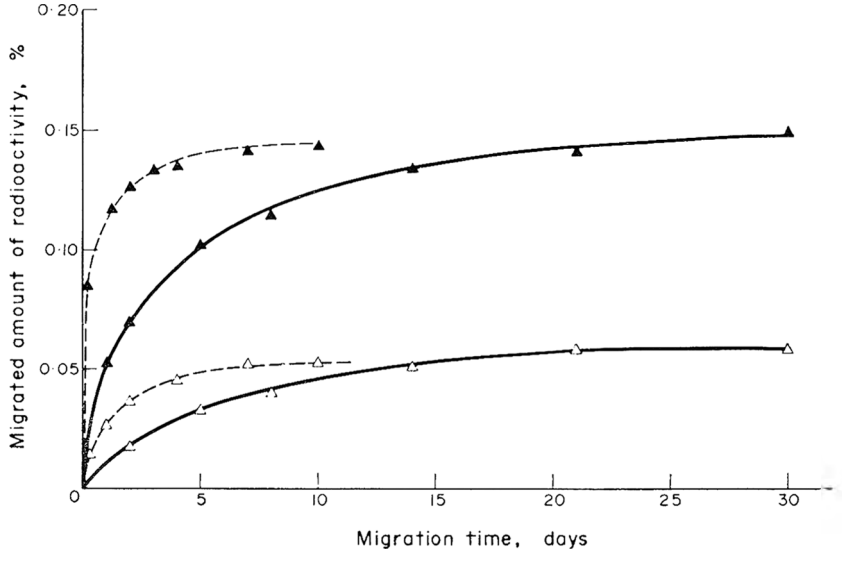

FIG. 4. Effect of contact time on the migration of radioactivity from PVC test films (Solvic, ▲; Vinoflex, △) containing ^{14}C-labelled tin stabilizer and maintained in one-sided contact with fat simulant HB 307 at 20°C (▲——▲; △——△) and 40°C (▲- - -▲; △- - -△). The amount of migrated radioactivity is expressed as a percentage of the radioactivity of the original test film.

Table 5. *Migration of plasticizer from PVC test films into varying volumes of fat simulant HB* 307

Content of Palatinol AH (%, w/w)	PVC test film*								Migration of additive (%)‡ with a depth of simulant between two films of		
	Specific radioactivity				Thickness		Weight				
	Palatinol AH-[^{14}C]		Film								
	μCi/g	s%† (n = 10)	μCi/g	s%† (n = 40)	μm	s%† (n = 50)	mg/dm²	s%† (n = 5)	5 mm	10 mm	20 mm
4·0	104·21	1·17	4·139	2·71	222	7·3	2990·3	3·0	0·019 (3·0)	0·017 (2·1)	0·009 (7·4)
6·0	70·92	1·11	4·225	1·24	224	11·2	2994·6	3·0	0·046 (2·4)	0·039 (1·1)	0·043 (2·7)
9·0	34·90	1·06	3·162	1·96	218	11·8	3003·2	2·8	0·58 (0·9)	0·51 (1·9)	0·58 (2·4)
12·0	34·90	1·06	4·155	1·57	220	7·0	2960·3	3·0	2·11 (1·0)	2·13 (1·4)	2·04 (0·8)
16·0	34·90	1·06	5·823	2·07	210	5·5	2758·5	2·7	9·07 (0·4)	8·73 (0·8)	8·30 (0·5)
20·0	34·90	1·06	6·878	2·24	216	5·6	2819·5	2·8	24·63 (0·7)	22·51 (0·9)	23·91 (1·6)
25·0	34·90	1·06	8·617	1·58	213	4·8	2849·1	2·7	82·47 (0·6)	79·76 (0·4)	72·48 (0·9)

*Composition: 95·0–74·0% (w/w) Vinoflex 503 (BASF, Ludwigshafen), 4·0–25·0% Palatinol AH (di-(2-ethyl-*n*-hexyl) [7,8-^{14}C]phthalate), 0·5% Wachs E (Farbwerke Hoechst, Frankfurt/Main), 0·5% Stabilizer C (Farbenfabriken Bayer, Leverkusen).

†$s\% = \dfrac{\text{standard deviation}}{\text{mean value of } n \text{ single values}} \times 100.$

‡Values in parentheses are the coefficients of variation (s%) of the single results about the mean of five determinations. Migration was determined after one-sided contact for 10 days at 40°C.

Palatinol AH in the simulant layers reached relatively high values. Nevertheless, the amounts of Palatinol AH migrating, for instance, from the PVC film containing 25% (w/w) of plasticizer into the different depths of fat simulant layers were approximately equal, and this similarity was even more marked with films containing lower levels of plasticizer. The curves in Fig. 5 are the means of the 15 individual determinations of migration from each test film plotted as a function of the plasticizer content and the coefficient of variation of the single results about the mean. The narrow range of error confirms that the amounts of plasticizer migrating from the PVC test films into HB 307 are practically independent of the depth of the simulant layer. The amounts of the antioxidant, Ionox 330, that migrated from the HD-polyethylene film into HB 307 were 0·084, 0·082 and 0·069% with simulant layers of 5, 10 and 20 mm, respectively.

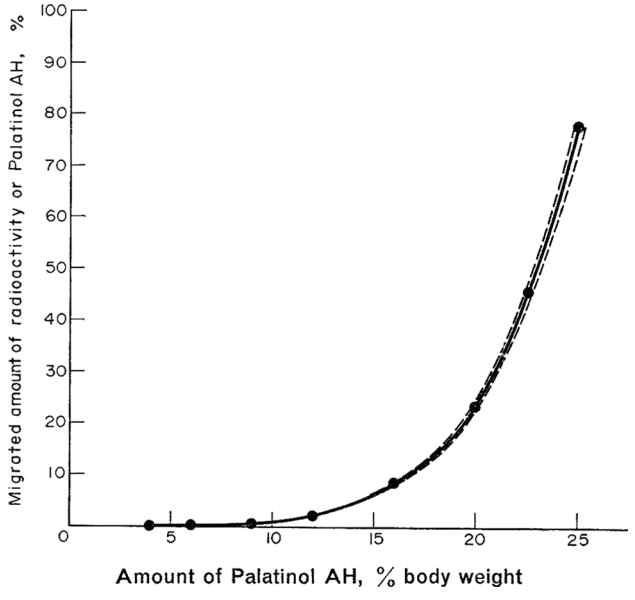

FIG. 5. Mean migration of plasticizer, Palatinol AH, from PVC test films as a function of the plasticizer content. Broken lines indicate the coefficient of variation of the single results about the mean.

We therefore consider that a simulant layer of 5 mm between two test films is sufficient for a migration test. Apart from savings on the cost of the simulant, the quantitative determination of migrated additives is made easier by increasing the ratio of contact area to the amount of simulant. In comparative migration tests, the contact areas between the test film and test fat should be kept constant throughout the study, since the amount of additive migrating in each case is proportional to this area (Strodtz & Henry, 1961). The design of the migration cell reflects normal conditions of use, in that the test film is in one-sided contact with the simulant. The final version of this migration cell provides a contact area between the test film and the fat simulant of 2×50 cm^2 and a simulant layer 5 mm in depth between the two circular films.

It may be concluded that for testing films used for packaging edible oils and foodstuffs containing fats, it is appropriate to test migration into fat simulant HB 307 (m.p. (clear) 29·3°C) in a migration cell providing a contact area of 2×50 cm^2, and a simulant layer 5 mm in depth. After the required amount of simulant has been placed in the cell, migration

is tested either for 10 days at 40°C or under conditions actually encountered in practice. For carrying out migration tests on plastics containers, the same fat simulant and test conditions may be used, and when its volume and internal surface have been determined, the container is filled with glass beads and the required amount of simulant is placed in it. Analysis of the simulant follows either type of contact and the resulting level of migrate in the simulant (μg/g) can be converted to μg migrate/dm^2 contact area and thence to μg migrate/g foodstuff.

It should be stressed that high temperatures encountered for limited periods in practice (e.g. during pasteurization, sterilization or preparation of foodstuffs in plastics packs) must be simulated by use of appropriate test temperatures and times. Moreover, experimental films made only for test purposes should be of the same thicknesses as the article that will actually be used, since the thickness of the film has a definite influence on the amount of additive migrating (Phillips & Marks, 1961). The extent of migration depends also on the physico-chemical properties of the test material, including its density, surface quality and crystallinity. Test specimens, both of films and plastics containers should therefore be made under standard production conditions.

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Effets de certaines variables sur la migration d'additifs des plastiques dans les graisses comestibles

Résumé—On a montré que des liquides organiques comme les alcanes et les éthers di-*n*-alcoyliques ne conviennent pas pour simuler la matière grasse. Un mélange de triglycérides synthétiques analytiquement purs, à distribution d'acides gras et de triglycérides similaire à celle de l'huile de coco, a été reconnu comme modèle universellement utilisable pour simuler des huiles comestibles et des aliments contenant de la graisse. Les auteurs ont étudié l'influence de la température sur la migration d'additifs à partir du CPV, du polyéthylène haute densité et du polystyrène dans des huiles comestibles et dans le modèle de matière grasse en question, le HB 307. Pour toutes les combinaisons de polymère et d'additif étudiées, la migration de l'additif dans le modèle de matière grasse a nettement augmenté quand la température d'essai dépassait 50°C. On a constaté que l'ampleur de cette augmentation et la température à laquelle elle commençait à se manifester dépendaient du type de plastique et des propriétés physiques et chimiques de l'additif. La quantité d'additif migrant est indépendante dans une large mesure de la profondeur de la couche de HB 307 (c'est-à-dire le rapport entre la surface de contact et la quantité de modèle de matière grasse), comme l'ont montré des essais avec des couches de ce modèle profondes de 2,5 à 10,0 mm. Ces résultats et d'autres, publiés ailleurs, ont servi à définir les conditions appropriées pour l'essai de films et de récipients de matière plastique destinés à l'emballage d'huiles comestibles et d'aliments gras.

Einfluss einiger Variabler auf die Wanderung von Zusätzen aus Kunststoffe in Speisefette

Zusammenfassung—Es wurde gezeigt, dass organische Flüssigkeiten wie Alkane und Di-*n*-alkyläther nicht zufriedenstellend für die Anwendung als Fettsimulanten sind. Ein synthetisches, analytisch reines Triglyceridgemisch, das eine Fettsäure- und Triglyceridverteilung ähnlich der von Kokosnussöl besitzt, hat sich als universell verwendbarer Simulant für Speisefette und fetthaltige Lebensmittel erwiesen. Der Einfluss der Temperatur auf die Wanderung von

Zusätzen aus PVC, Hochdruckpolyäthylen und Polystyrol in Speiseöle und in diesen Fettsimulanten, HB 307, wurde untersucht. Bei allen untersuchten Polymer/Zusatz-Kombinationen nahm die Zusatzstoffwanderung in den Fettsimulanten oberhalb der Versuchstemperatur von 50°C deutlich zu. Die Stärke dieser Zunahme und die Temperatur, bei der sie in Erscheinung trat, erwiesen sich als abhängig vom Typ des Kunststoffmaterials und den physikalisch-chemischen Eigenschaften des Zusatzstoffes. Die Menge des wandernden Zusatzstoffs ist weitgehend unabhängig von der Tiefe der Schicht aus HB 307 (d.h. dem Verhältnis der Kontaktfläche zur Menge des Simulanten), wie Untersuchungen mit Simulansschichten unterschiedlicher Dicke zwischen 2,5 und 10,0 mm ergaben. Diese Ergebnisse und andere, die an anderen Stellen veröffentlicht wurden, sind zur Definition von Bedingungen benutzt worden, die sich für die Prüfung von Kunststofffilmen und -behältern zur Verpackung von Speiseölen und fetthaltigen Lebensmitteln eignen.

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Prolonged Administration of *Penicillium viridicatum* to Mice: Preliminary Report of Carcinogenicity

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Abstract—The long-term toxic effects and carcinogenicity of a toxigenic species of *Penicillium viridicatum* were studied by feeding mice on a rice culture of the fungus at concentrations of 5·0 and 7·5% in the diet for 52–55 wk. A control group was fed only the purified diet. In mice fed the 7·5% concentration, the incidence of pulmonary tumours was about 57% higher than that in control mice. The tumours were classified as adenomas or adenocarcinomas arising from the bronchial and alveolar epithelium. The numbers of mice with adenomatosis and adenoma were four of 41 controls, 67 of 98 fed the 7·5% fungal diet and eight of 91 fed the 5% fungal diet. Adenocarcinoma occurred in 17 of the 98 mice given the 7·5% fungal diet. The incidence of other neoplasms was not affected by the feeding of the fungal culture. The high incidence of pulmonary neoplasms in mice fed the 7·5% fungal diet appeared to be directly related to the feeding of the culture of *P. viridicatum*. The possible presence of certain mycotoxins, including the aflatoxins and sterigmatocystin, was excluded by chemical analysis. It was concluded that an unidentified toxin and an unidentified carcinogen were present in the culture of *P. viridicatum*, and that these substances might be the same compound or separate chemical entities.

INTRODUCTION

Mycotoxins have received much attention in the past 20 years because of concern for these dietary contaminants as causes of neoplastic and non-neoplastic diseases in man and animals. In 1960–1961, outbreaks in different parts of the world of diseases affecting fish (Ashley & Halver, 1961; Levaditi, Besse, Vibert & Nazimoff, 1960; Rucker, Yasutake & Wolf, 1961; Wood & Larson, 1961) and poultry (Asplin & Carnaghan, 1961; Blount, 1961), especially "turkey X" disease in England associated with aflatoxin-contaminated peanut meal, provided a great stimulus for research on mycotoxins and associated diseases. Reports of neoplastic hepatic lesions in trout in the United States (Ashley & Halver, 1961; Rucker *et al.* 1961; Wood & Larson 1961) and in France (Levaditi *et al.* 1960) were associated with the feeding of a commercial diet that contained aflatoxin.

The penicillia are ubiquitous fungi and certain species are known to invade feedstuffs and elaborate toxic metabolites. Among the mycotoxins produced by penicillia are three—patulin (Dickens & Jones, 1961), penicillin G (Dickens & Jones, 1961, 1963 & 1965) and

penicillic acid (Dickens & Jones, 1961 & 1963)—that have produced sarcomas at sc injection sites in mice and rats. Luteoskyrin and islanditoxin, produced by *P. islandicum*, have induced hepatoma when fed to mice and rats (Miyake & Saito, 1965), and the feeding of griseofulvin was followed by the development of hepatomas in mice (Hurst & Paget, 1963).

This study was undertaken to characterize the effects of long-term feeding of a toxic rice culture of an Indiana isolate of *P. viridicatum* to male and female white Swiss mice.

EXPERIMENTAL

ICR white Swiss mice of both sexes, obtained from Harlan Small Animal Industry, Cumberland, Ind., were divided into three experimental groups, comprising a control group of 21 males and 20 females fed only the purified diet, obtained from General Biochemicals Corp., Chagrin Falls, Ohio (Mills & Murray, 1960), a group of 64 males and 34 females fed this diet containing a 7·5% concentration of a rice culture of *P. viridicatum* and a group of 42 males and 49 females fed the same diet containing 5% of the fungal culture. The 7·5% fungal diet was fed for periods of 2 wk alternating with 2 wk on the control diet. This schedule was adopted because previous studies had indicated that mice would not survive continuous feeding on a 7·5% fungal diet. The numbers of male and female mice assigned to the respective diets were unequal because of errors in sexing the weanlings. The fungal culture was prepared as described by Carlton & Tuite (1970). The mice were started on the experiment as weanlings, approximately 3 wk of age and with body weights between 12 and 15 g.

Mice were housed in groups of five with the sexes separated, in plastics shoebox-type cages with a stainless-steel top and dry sterile bedding. Water and feed were provided *ad lib*. The cages were located in the rooms of an air-conditioned animal house with a room temperature between 70 and 75°F.

Mice were weighed weekly for the first month and then at monthly intervals for the remainder of the 1-yr test period. Observations of the general health of the mice were made daily. During the experimental period, some of the mice from several groups either died or became moribund and the latter were killed for autopsy. At termination, mice were killed by cervical dislocation for autopsy, and tissues including the brain, eyes, lungs, heart, liver, kidney, spleen, stomach and intestines, trachea, testes and bone were fixed in 10% buffered formalin. Portions of these organs and multiple sections of each lung were prepared for paraffin sectioning and the sections were stained with haematoxylin and eosin.

The pulmonary alterations were evaluated microscopically and placed into three categories, adenomatosis, adenoma and adenocarcinoma, according to criteria presented by Amaral-Mendes (1969).

RESULTS

Rough hair, weight loss and dehydration were seen in several mice fed the test diets for 3–4 wk. Many of the affected mice recovered after a few weeks, but other mice died.

The mortality and incidence of pulmonary tumours are given in Table 1. There was no great difference between male and female mice in the incidence of pulmonary tumours, and the results for both sexes will therefore be discussed together. The tumour incidence was low in control mice (4/41) and in each case was a single pulmonary nodule that was considered on histological evidence to be an adenoma.

Table 1. *Mortality and incidence of pulmonary tumours in mice fed diets containing a rice culture of* P. viridicatum

Dietary level of fungal culture (%)	Time of appearance of first tumour (days)	Mortality by day 385		Mice killed at termination		Total no. of mice with tumours
		Total deaths*	No. with tumours	Total	No. with tumours†	
		Males				
0 (control)	384	2/21	0	19	2/19	2
5·0	293	21/42	1	19	3/21	4
7·5	194	22/64	9	42	36/42	45
		Females				
0 (control)	386	1/20	0	19	2/19	2
5·0	368	14/49	0	35	4/35	4
7·5	329	16/34	4	18	18/18	22

* No. observed/total no. of mice.
† No. of tumour-bearing mice/no. killed.

Among mice fed the 7·5% fungal diet, the first pulmonary tumour was seen in a male mouse after 194 days of feeding. The overall incidence of pulmonary tumours was about 67% (67/98 mice). In most of these mice, the pulmonary tumours were multiple. In 17 of the mice with tumours at least one was classified morphologically as an adenocarcinoma (Table 2).

Among mice fed the 5·0% fungal diet, the first pulmonary tumour was seen in a male mouse after 293 days of feeding. The incidence of pulmonary tumours in this group (8/91) did not differ from that in the controls.

Table 2. *Incidence of neoplasms in mice fed diets containing a rice culture of* P. viridicatum

Dietary level of fungal culture (%)	Pulmonary alterations		
	Adenomatosis	Adenoma	Adenocarcinoma
	Males		
0 (control)	0/21	2/21	0/21
5·0	4/42	4/42	0/42
7·5	45/64	45/64	10/64
	Females		
0 (control	3/20	2/20	0/20
5·0	4/49	4/49	0/49
7·5	22/34	22/34	7/34

In each case, incidence is expressed as no. of mice affected/total no. on test.

Except for the two control males, mice with pulmonary adenoma also showed alterations indicative of adenomatosis (Table 2). Adenomatosis was characterized by small sub-pleural or deep parenchymal foci of cuboidal hyperplastic and hypertrophied alveolar epithelial cells (Figs 1 & 2). In these foci, the alveoli were encroached upon and some were obliterated.

There was no evidence of a host reaction. These foci were irregular in shape and variable in size and did not compress adjacent pulmonary tissue.

The pulmonary adenomas (Figs 3 & 4) were frequently seen as firm, tan-white, raised nodules, varying in size from approximately 2 to 6 mm in diameter. They occurred in any lobe of the lung and were visible on both the dorsal and ventral surfaces.

Adenomas (Figs 3 & 4) occurred in both sub-pleural and deep parenchymal locations. Occasionally, their location near bronchioles suggested a bronchiolar origin, but most often they appeared to arise in the pulmonary parenchyma without any obvious association with the airways. The adenomas had an occasional mitotic figure.

Some male and female mice fed the 7·5% fungal diet developed pulmonary adenocarcinoma (Figs 5 & 6). In these neoplasms, marked proliferation of large polygonal epithelial cells with frequent mitoses resulted in extensive invasion of the adjacent pulmonary tissue, with obliteration of the normal architecture.

The other neoplasms found in this study were a myelosarcoma in one female on the 7·5% fungal diet and in another on the 5·0% diet, renal tubular adenomas in four males and one female on the 7·5% diet, a malignant lymphoma in a male on the 7·5% diet, hepatoma in one male and one female on the 7·5% and in one female on the 5% diet and a mammary gland adenocarcinoma in one female control animal. The incidence of these tumours was not apparently influenced by the feeding of the fungal diets.

DISCUSSION

Neoplasms of the lung occurred in all groups, but the incidence was great only in the group fed the 7·5% fungal diet. In this study, no attempt was made to separate the several tumours on the basis of their derivation from bronchial or alveolar epithelial cells. Others, including Grady & Stewart (1940), have studied the histogenesis of pulmonary neoplasms in mice and have concluded that most of the pulmonary neoplasms arise from alveolar epithelial cells. Shimkin (1955) and Stewart (1953) observed that there was essentially no difference in histological appearance between spontaneous and induced tumours, between tumours in different strains of mice or between tumours induced by different kinds of carcinogen.

It has been suggested by Shimkin (1955) and by Stewart (1953) that the development and incidence of pulmonary tumours in mice are determined by the genetic constitution of the mouse strain and that genetically susceptible mice can develop a high incidence of neoplasms of the lungs when exposed to a variety of carcinogenic stimuli. The high incidence, about 67%, of pulmonary tumours in mice fed the 7·5% fungal diet for 1 yr appears to be due to a carcinogenic substance elaborated by the fungus acting in a genetically susceptible host. That a carcinogen was present in the fungal culture can be deduced from at least two lines of evidence. First, the incidence of pulmonary tumours was very low (about 10%) in the controls; and secondly, others (Snell & Stewart, 1962; Tannenbaum, 1965) have observed that neoplasms of the lungs are uncommon in most strains of mice during the first year of life.

Definite evidence is not at present available on the specific carcinogen responsible for the increased incidence of pulmonary tumours in the mice fed the 7·5% fungal diet, but such common mycotoxins as aflatoxins, ochratoxin A and sterigmatocystin were eliminated by chemical analysis.

The interaction between an oncogenic virus in the lungs of the test mice and an unidenti-

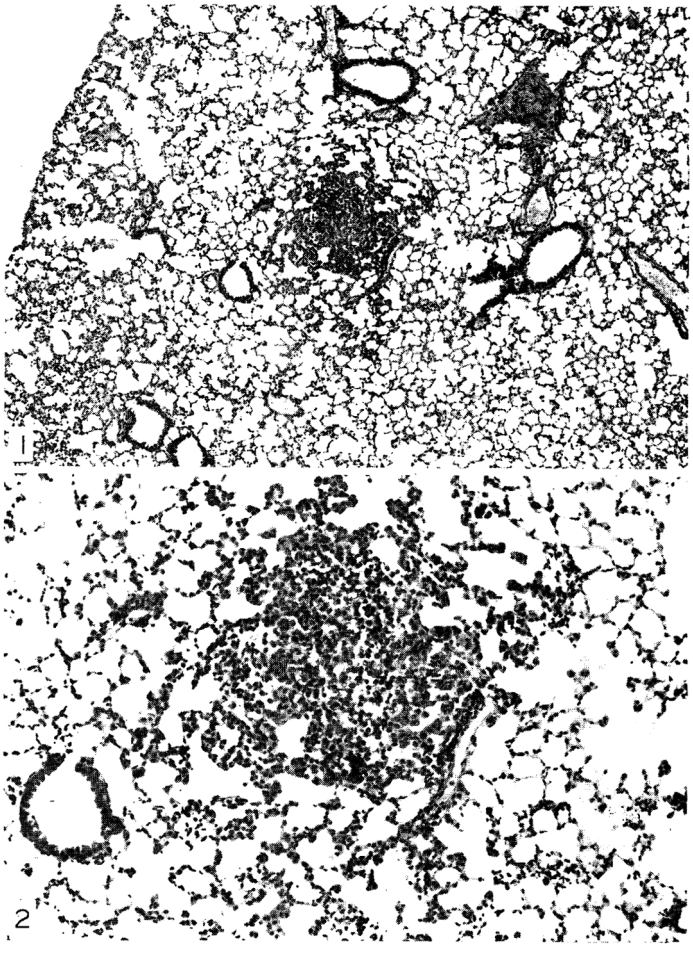
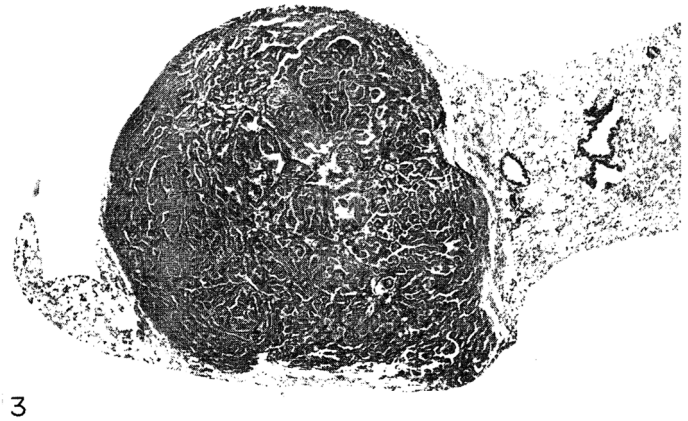


FIG. 1. Focal adenomatosis in the lung of a mouse fed the 7·5% fungal diet. Haematoxylin and eosin ×56,
FIG. 2. Higher magnification of Fig. 1, illustrating the proliferation of alveolar septal epithelium and the absence of inflammatory cells. Haematoxylin and eosin ×140.



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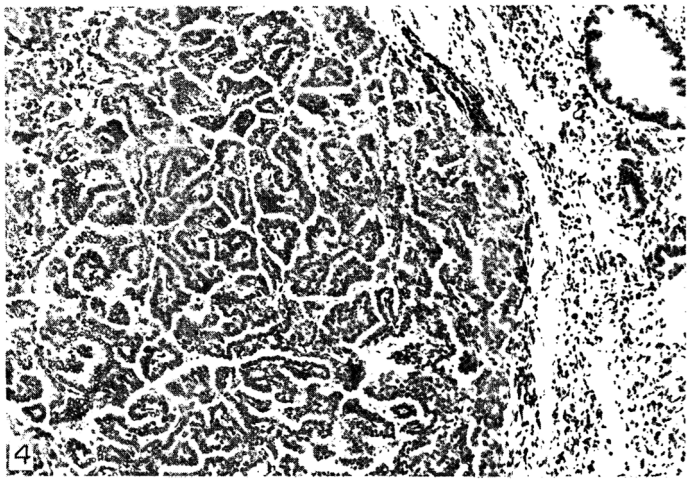

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FIG. 3. Adenoma of the peripheral lung of a mouse fed the 7·5% fungal diet. Haematoxylin and eosin ×35.

FIG. 4. A higher magnification of Fig. 3, illustrating the alveolar arrangement of neoplastic epithelial cells. Haematoxylin and eosin ×140.

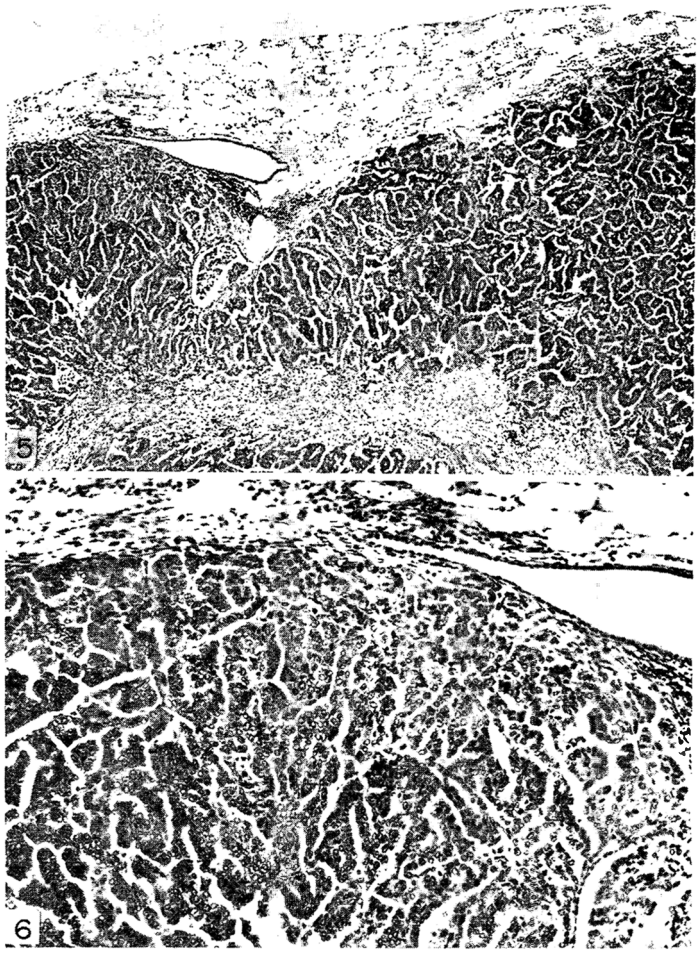

FIG. 5. Adenocarcinoma in the lung of a mouse fed the 7·5% fungal diet. Haematoxylin and eosin ×35.

FIG. 6. Higher magnification of Fig. 5, illustrating the densely cellular and alveolar arrangement of the cells and the invasion of a small bronchiole. Haematoxylin and eosin ×140.

fied carcinogen in the fungal culture can be considered a possible cause of the increased incidence of pulmonary tumours in mice fed the 7·5% fungal diet. No attempts were made to isolate viruses or detect antibodies to murine viruses. There was serological evidence that the commercial colony was not infected with the viruses of mouse hepatitis, ectromelia, polyoma, lymphocytic choriomeningitis or the pneumotropic and Sendai viruses (Harlan Industries, Cumberland, Ind., personal communication 1972).

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Administration prolongée de *Penicillium viridicatum* à des souris—Rapport préliminaire sur le pouvoir carcinogène

Résumé—Pour étudier les effets toxiques et carcinogènes à long terme d'une espèce toxigène de *Penicillium viridicatum*, on a fait consommer une culture sur riz de ce champignon par des souris pendant 52–55 semaines et à raison de 5,0 à 7,5% du régime. Un groupe témoin ne recevait que l'aliment purifié. La fréquence de tumeurs pulmonaires a été plus élevée d'environ 57% chez les souris soumises au régime à 7,5% que chez les souris témoins. Les tumeurs étaient des adénomes ou des adénocarcinomes de l'épithélium des bronches et des alvéoles. Les cas d'adénomatose et d'adénome ont été au nombre de 4 chez 41 souris témoins, de 67 chez 98 souris soumises au régime à 7,5% et de 8 chez 91 souris du groupe à 5%. Des 98 souris du groupe à 7,5%, 17 ont contracté un adénocarcinome. La consommation de culture mycologique n'a pas influé sur la fréquence d'autres néoplasmes. Il y avait un rapport direct entre le régime à 7,5% et la grande fréquence de néoplasmes pulmonaires. L'analyse chimique ayant exclu la présence de certaines mycotoxines, en l'occurrence les aflatoxines et la stérigmatocystine, on conclut que la culture de *P. viridicatum* recélait une toxine et un carcinogène non identifiés, lesquels peuvent être soit la même substance, soit des entités chimiques différentes.

Längere Verabreichung von *Penicillium viridicatum* an Mäuse: Vorläufiger Bericht über die Carcinogenität

Zusammenfassung—Die toxischen Effekte und die Carcinogenität nach längerer Verabreichung einer toxinhaltigen Spezies von *Penicillium viridicatum* wurden mittels Verfütterung einer Reiskultur des Pilzes mit Konzentrationen von 5,0 und 7,5% im Futter an Mäuse über 52–55 Wochenuntersucht. Eine Kontrollgruppe erhielt nur das gereinigte Futter. Bei Mäusen, welche Futter mit der Konzentration 7,5% erhielten, war die Häufigkeit pulmonaler Tumoren etwa 57% höher als bei Kontrolltieren. Die Tumoren wurden als Adenome oder Adenocarcinome klassifiziert, die aus dem bronchialen und alveolaren Epithel entstanden. Die Zahl der Mäuse mit Adenomatosis und Adenom betrug 4 von 41 Kontrolltieren, 67 von 98, welche das Futter mit 7,5%, und 8 von 91, welche das Futter mit 5% Pilzkultur erhalten hatten. Adenocarcinome traten bei 17 der 98 Mäuse auf, welche das Futter mit 7,5% Pilzkultur erhalten hatten. Die Häufigkeit anderer Neoplasmen wurde durch die Verfütterung des Futters mit Pilzkultur nicht beeinflusst. Die grosse Häufigkeit von pulmonalen Neoplasmen bei Mäusen, die Futter mit 7,5% Pilzkultur erhalten hatten, schien in direkter Beziehung zur Verfütterung der Kultur von *P. viridicatum* zu stehen. Die mögliche Anwesenheit bestimmter Mycotoxine einschliesslich der Aflatoxine und Sterigmatocystin wurde durch chemische Analyse ausgeschlossen. Es wurde der Schluss gezogen, dass ein unidentifiziertes Toxin und ein unidentifiziertes Carcinogen in der Kultur von *P. viridicatum* vorhanden waren und dass diese Substanzen die gleiche Verbindung oder verschiedene chemische Individuen sein könnten.

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Inhibition of Muscle Aldolase by Penicillic Acid and Patulin *In Vitro**

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Abstract—The mycotoxins, penicillic acid and patulin, were found to inhibit aldolase non-competitively *in vitro* with inhibition constants (K_i) of $9{\cdot}0 \times 10^{-6}$ M and $1{\cdot}3 \times 10^{-5}$ M, respectively. The inhibition was not prevented by the addition of cysteine to the assay system. The cysteine adduct of penicillic acid was comparable to the original mycotoxin in its inhibition of aldolase. However, less inhibition was observed when the cysteine adduct of patulin was used. Chemical analyses of the inactivated enzyme revealed that with each mycotoxin covalent interaction with $-SH$ and $-NH_2$ groups in the protein had occurred.

INTRODUCTION

Penicillic acid and patulin are fungal toxins with an α,β-unsaturated lactone structure produced by several species of Aspergillus and Penicillium (Abraham & Florey, 1949). Both toxins exhibit antibiotic activity and are toxic to animals (Ciegler, Mintzlaff, Weisleder & Leistner, 1972; Norstadt & McCalla, 1963; Oxford, 1942; Waksman, Horning & Spencer, 1943) and their presence in foods and feeds for human and animal consumption has therefore been considered hazardous. Nevertheless, thiols inactivate both mycotoxins by reacting by Michael addition, with the double bond conjugated to the lactone carbonyl group (Cavins & Friedman, 1968; Ciegler *et al.* 1972; Dickens & Jones, 1961; Geiger & Conn, 1945; Jones & Young, 1968). Penicillic acid is reported to interact also with amino groups (Ciegler *et al.* 1972). Recently, the inhibition of two thiol enzymes, alcohol and lactic dehydrogenases, by both mycotoxins has been demonstrated (Ashoor & Chu, 1973). Since rabbit-muscle aldolase loses its activity when some of the lysine and cysteine residues are chemically modified (Horecker, Tsolas & Lai, 1972), the possibility of studying the effect of penicillic acid and patulin on the activity of this enzyme was brought to our attention. This paper presents evidence concerning the inhibitory effect of these mycotoxins on this enzyme as well as the interactions of these mycotoxins with the amino acid residues in the active sites of the protein.

EXPERIMENTAL

Preparation of toxins. Penicillic acid was produced in rice by *Penicillium cyclopium* NRRL 1888 and was purified according to the method of Bentley & Keil (1962). Patulin

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was kindly supplied by Dr. T. M. McCalla, of the University of Nebraska, and was further recrystallized from ethyl ether. The purity of both toxins was verified by thin-layer chromatography, melting point and spectrophotometric and mass spectroscopic analyses. Freshly prepared aqueous solutions of appropriate concentration were used for the inhibition studies. Molar absorptivity of $1{\cdot}4 \times 10^4$ at 222 nm for penicillic acid and of $2{\cdot}0 \times 10^4$ at 276 nm for patulin were used to calculate the toxin concentration in certain cases.

Enzyme assay. Rabbit-muscle aldolase, the tetrasodium salt of fructose 1,6-diphosphate and hydrazine sulphate were purchased from Sigma Chemical Co., St. Louis, Mo. Aldolase was assayed by the hydrazine method of Jagannathan, Singh & Damondaran (1956). Since penicillic acid hydrazone does not absorb appreciably at the wavelength used in this assay, the interference due to such absorption was considered irrelevant (Kurtzman & Ciegler, 1970). The enzyme concentration was determined using an extinction coefficient $E^{0.1\%}_{1\,cm}$ of 0·91 at 280 nm (Tayler & Lowrey, 1956), and assuming a molecular weight of 160,000 (Sia & Horecker, 1968). The inhibitory effect of the toxins was determined by incubating an appropriate amount with a known amount of the enzyme for 1 hr at 37°C. Initial velocities of the mycotoxin-treated enzyme and of the controls were then determined. The Michaelis constant (K_m), the inhibition constant (K_i) and the type of inhibition were determined by the method of Lineweaver & Burk (1934).

Effect of cysteine on the inactivation of aldolase by mycotoxins. Two approaches were used. In the first, enough cysteine was added to each incubation mixture containing mycotoxin, substrate and enzyme, to give a final concentration of $3{\cdot}3 \times 10^{-4}$ M, and the mixture was then incubated for 20 min at 37°C before the enzyme activity was assayed as mentioned above. In the second, a mycotoxin–cysteine adduct was prepared by reacting either penicillic acid or patulin with cysteine (in a molar ratio of 1:2) at 37°C for 2 hr. An appropriate amount of this adduct was added to the incubation mixture in the presence or absence of cysteine, and the enzyme activity was determined.

Chemical analyses of the partially inactivated enzyme. An appropriate amount of aldolase ($1{\cdot}25 \times 10^{-5}$ M) was reacted with either penicillic acid ($4{\cdot}10 \times 10^{-3}$ M) or patulin ($6{\cdot}2 \times 10^{-3}$ M) at 37°C for 1 hr at pH 8·6 in 0·05 M-Tris buffer. After reaction, one half of each solution was reduced with $NaBH_4$ according to Lai, Tchola, Cheng & Horecker (1965). Two-ml solutions, along with a control, were dialysed against 2 litres of distilled water at 6°C, with the outer water being changed twice daily, throughout a period of 72 hr. The enzyme activity, free —SH groups (Ellman, 1959) and free $-NH_2$ groups (Moore & Stein, 1948) of the mixtures were then determined. The dialysed mixtures were also spotted on thin-layer chromatographic plates (0·3 mm, Adsorbosil-5 from Applied Science Laboratories, Inc., State College, Penn.) and developed with chloroform–methanol (90:10, v/v). The developed plates were exposed to concentrated ammonia fumes for 5 min, and the fluorescent spots were located under ultraviolet light.

RESULTS AND DISCUSSION

The inhibitory effects of varied amounts of penicillic acid and patulin on aldolase activity at a constant substrate concentration are presented in Fig. 1. The concentration of penicillic acid required for 50% inhibition of aldolase was $9{\cdot}5 \times 10^{-6}$ M, compared with a $1{\cdot}3 \times 10^{-5}$ M concentration of patulin. The molar ratio of toxin to aldolase causing 50% inhibition was approximately 127 for penicillic acid and 173 for patulin. Lineweaver–Burk plots, shown in Fig. 2, indicate that both penicillic acid and patulin inhibit muscle aldolase non-

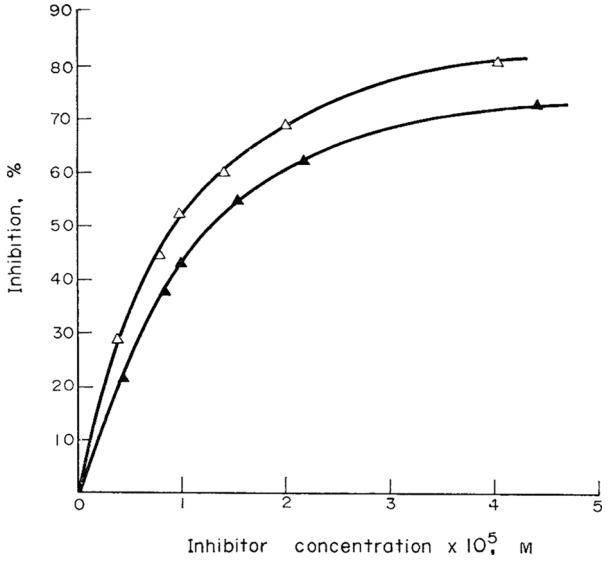

FIG. 1. Effect of varying concentrations of patulin (▲——▲) and penicillic acid (△——△) on the activity of muscle aldolase in 0·05 M-Tris buffer at pH 8·6 and 37°C for 20 min. The concentrations of enzyme, fructose 1,6-diphosphate and hydrazine sulphate (in $1{\cdot}0 \times 10^{-4}$ M-EDTA) were $7{\cdot}45 \times 10^{-8}$ M, $3{\cdot}6 \times 10^{-3}$ M and $3{\cdot}5 \times 10^{-3}$ M, respectively.

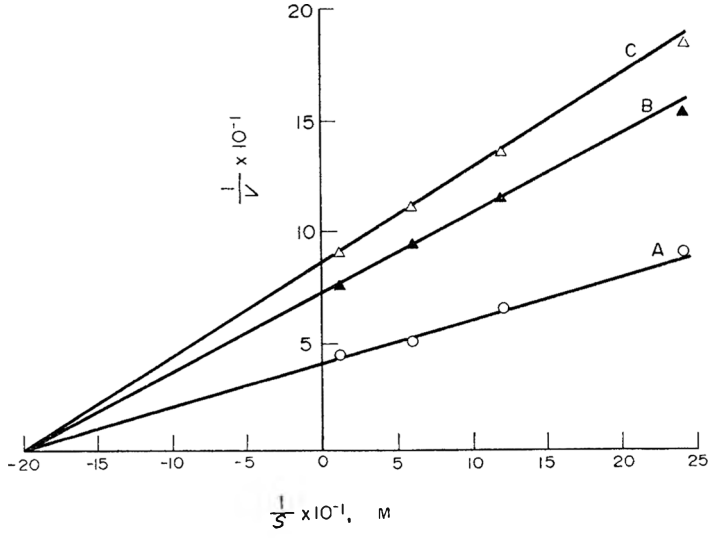

FIG. 2. Lineweaver plots for aldolase, showing control reaction (○——○), reaction inhibited by $1{\cdot}0 \times 10^{-5}$ M-patulin (▲——▲) and reaction inhibited by $1{\cdot}0 \times 10^{-5}$ M-penicillic acid (△——△). The reaction conditions were as indicated in Fig. 1, except that the fructose 1,6-diphosphate concentration varied from $3{\cdot}6 \times 10^{-4}$ to $7{\cdot}2 \times 10^{-3}$ M.

competitively. The K_m value for the substrate and the K_i values for penicillic acid and patulin were found to be $5{\cdot}0 \times 10^{-3}$ M, $9{\cdot}0 \times 10^{-6}$ M and $1{\cdot}3 \times 10^{-5}$ M, respectively. These results suggest that both mycotoxins have higher affinity for the enzyme than does the substrate fructose 1,6-diphosphate, and hence can be considered as effective inhibitors. The affinity of penicillic acid for the enzyme appears to be slightly higher than that of patulin.

The effect of cysteine on the inactivation of aldolase by the mycotoxins is shown in Table 1. Addition of cysteine at a concentration 8–16 times greater than that of the mycotoxins restored only a small part of the enzyme activity. Cysteine–penicillic acid adduct inhibited the enzyme to the same degree as an equivalent amount of penicillic acid alone. However, the cysteine–patulin adduct inhibited the enzyme to a much lesser extent than an equivalent amount of patulin. In order to determine the nature of the binding and the groups in the enzyme that reacted with either mycotoxin, the $-NH_2$ groups and $-SH$ groups in

Table 1. *Effect of cysteine, mycotoxins and cysteine–mycotoxin adducts on aldolase activity*

Incubation mixture*	Relative enzyme activity (%)
Control	100
Cysteine	100
Penicillic acid	36
Penicillic acid + cysteine	42
Patulin	26
Patulin + cysteine	33
Cysteine–penicillic acid adduct	32
Cysteine–penicillic acid adduct + cysteine	38
Cysteine–patulin adduct	68
Cysteine–patulin adduct + cysteine	70

*The assays were carried out under the conditions described in the text with an enzyme concentration of $7{\cdot}45 \times 10^{-8}$ M in all experiments. The concentration of cysteine was $3{\cdot}3 \times 10^{-4}$ M; those of the inhibitors were as follows: penicillic acid, $2{\cdot}0 \times 10^{-5}$ M; patulin, $4{\cdot}0 \times 10^{-5}$ M; cysteine–penicillic acid adduct, $2{\cdot}0 \times 10^{-5}$ M; cysteine–patulin adduct, $4{\cdot}0 \times 10^{-5}$ M.

Table 2. *Modification of $-NH_2$ and $-SH$ groups in aldolase by penicillic acid and patulin*

Reaction	Subsequent reduction with $NaBH_4$	Relative activity (%)	$-NH_2$ groups modified* (mol/mol enzyme)	$-SH$ groups modified† (mol/mol enzyme)
Control	—	100	0	0
Aldolase + penicillic acid	—	30	12	10
	+	21	16	11
Aldolase + patulin	—	28	9	8
	+	22	14	9

*A total of 106 lysine residues plus 4 *N*-terminal amino groups/mol enzyme was used in the calculation (Lai, 1968).

†A total of 32 cysteine residues/mol enzyme was used in the calculation (Lai, 1968).

the partially inactivated enzymes were measured after exhaustive dialysis. The results are presented in Table 2. It is clear that interaction of mycotoxins with the $-NH_2$ and $-SH$ groups in the enzyme had occurred. Thin-layer chromatographic analysis revealed that the mycotoxin fluoresence was associated with the protein spot at the origin. No free mycotoxin was observed in the reaction mixture. The results suggest that these mycotoxins were covalently bound to the enzyme.

Evidence has accumulated for the presence of two different groups of lysine residues plus some essential $-SH$ groups in the active site of muscle aldolase (Horecker *et al.* 1972). The inhibition of aldolase by penicillic acid and patulin observed in the present study may have been due to the interaction of these mycotoxins with some of the functional groups in the enzyme. The loss of enzyme activity was concomitant with a decrease in free $-SH$ and $-NH_2$ groups in the enzyme (Table 2). Assuming that the four *N*-terminal amino groups were all affected, approximately 8–10 cysteine and 5–8 lysine residues for each mole of enzyme were modified by the mycotoxins. Reducing the enzyme-mycotoxin mixtures with $NaBH_4$ resulted in no further modification of cysteine. However, the number of lysine residues modified increased to 10–12 after reduction. Apparently both mycotoxins reacted with lysine residues by two different types of reaction, addition and Schiff-base formation. The addition reaction caused the major loss of enzyme activity (Table 2). The rate of the addition reaction was somewhat higher with penicillic acid, a fact which may explain the slightly higher affinity of this mycotoxin for the enzyme.

The finding that cysteine did not prevent the inhibitory effect of either mycotoxin, while cysteine adducts of the mycotoxin also displayed an inhibitory effect on the enzyme, suggests that the primary cause of such inhibition may indeed be due to interaction between the ϵ-amino group of lysine residues in the active centre of the enzyme and the carbonyl group of the lactone ring of either mycotoxin. The role of cysteine residues in enzyme activity becomes apparent from the observation that the cysteine adduct of patulin inhibited the enzyme to a much smaller extent than did patulin, penicillic acid or the cysteine adduct of penicillic acid. The fact that the cysteine adduct of penicillic acid still had a markedly inhibitory effect on the enzyme may have been because the two reaction sites of penicillic acid did not both react with added cysteine, thus leaving an available site to react with the enzyme.

The present study has demonstrated the covalent interaction of each mycotoxin with the $-NH_2$ and $-SH$ groups in aldolase, causing inactivation of the enzyme. However, there is a need for further studies of the exact reaction site(s), including, for example, the isolation of lysine- and cysteine-mycotoxin adducts from the inactivated enzyme, as well as the study of amino acid composition and the sequence of mycotoxin-containing peptide(s).

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Inhibition *in vitro* de l'aldolase du muscle par l'acide pénicillique et la patuline

Résumé—On a constaté que deux mycotoxines, l'acide pénicillique et la patuline, inhibent l'aldolase *in vitro*. Ces inhibitions sont non-compétitives et les constantes d'inhibition (K_i) respectives sont $9{,}0 \times 10^{-6}$ M et $1{,}3 \times 10^{-5}$ M. L'addition de cystéine au milieu de détermination n'a pas empêché l'inhibition. Le systéme cystéine + acide pénicillique a inhibé l'aldolase de façon comparable à celle de la mycotoxine originelle, mais on a observé moins d'inhibition avec le système cystéine + patuline. Les analyses chimiques de l'enzyme inactivé ont révélé qu'il s'était produit, avec chaque mycotoxine, une interaction covalente avec les radicaux —SH et $—NH_2$ de la protéine.

Inhibierung von Muskelaldolase durch Penicillinsäure und Patulin *in vitro*

Zusammenfassung—Die Mycotoxine Penicillinsäure und Patulin hemmen *in vitro* nicht kompetitiv die Aldolase *in vitro* mit der Hemmkonstante (K_i) $9{,}0 \times 10^{-6}$ M bzw. $1{,}3 \times 10^{-5}$ M. Die Hemmung wurde durch die Zugabe von Cystein zu dem Versuchsansatz nicht verhindert. Das Cysteinanlagerungsprodukt von Penicillinsäure war in der Inhibierung von Aldolase mit dem ursprünglichen Mycotoxin vergleichbar. Es wurde jedoch eine geringere Inhibition beobachtet, wenn das Cysteinaddukt von Patulin verwendet wurde. Die chemischen Analysen des inaktivierten Enzyms zeigen, dass mit jedem Mycotoxin kovalente Wechselwirkungen mit SH- und NH_2-Gruppen in dem Protein aufgetreten waren.

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Fate and Toxicity of Orally Administered Polyethylene Polyphosphonates*

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Abstract—The possible toxic effects of organic polymeric polyphosphonates, which are of potential use in the prophylaxis of dental caries and calculus, have been investigated. Uptake of these polymers from the gut, studied with the use of ^{32}P-labelled materials, is extremely low—less than $2{\cdot}5 \times 10^{-5}$% of the ingested dose. Subacute toxicity tests on rats fed a level of polyethylene polyphosphonate in the diet providing an intake of about 50 mg/kg/day, and acute toxicity tests in mice and rats given a single oral dose of up to 10 g/kg did not provide evidence of any significant detrimental effects. The low toxicity observed is explained by the extremely low absorption of these compounds into the body.

INTRODUCTION

Polyethylene polyphosphonate was shown to exhibit significant anticariogenic activity in rats (Anbar, St. John & Elward, 1973) as predicted from its effects on the behaviour of tooth enamel *in vitro* (Anbar, St. John & Scott, 1973). The effect of polymeric polyphosphonates on teeth seems to parallel that of organic oligophosphonates and primarily of the diphosphonates (Muhlemann, Bowles, Schait & Bernimoulin, 1970; Regolati & Muhlemann, 1970; Sturzenberger, Swancar & Reiter, 1971). However, the use of diphosphonates for dental prophylactic treatment may pose serious toxicological problems in view of their diverse physiological effects (Casey, Casey, Fleisch & Russell, 1972; Fleisch & Russell, 1972; Gasser, Morgan, Fleisch & Richelle, 1972). Polyalkyl polyphosphonates administered iv were shown to have similar effects on bone development (Anbar, Feldman, Lindley & Wolf, 1971a; Anbar, Feldman & Wolf, 1971b), but the large difference in molecular weight and molecular charge between organic oligophosphonates and polyphosphonates suggested that the latter would not be absorbed from the gut and would, therefore, be non-toxic if ingested. Because the use of these materials for dental treatment would involve topical application, most probably in the form of an additive to a dentifrice, oral toxicity may be the crucial difference between oligophosphonates and polymeric polyphosphonates. In this study, we investigated both the rate of uptake of polymeric phosphonates through the gut and the overall toxicity of these materials when ingested. The polymeric polyphosphonates were found to be non-toxic by this route, the LD_{50} being >10 g/kg. This was not surprising in view of the fact that their uptake from the gut was less than 0·25 ppm.

Three types of experiment were carried out within the framework of this study. In the first series, ^{32}P-labelled phosphonated polyethylene was used to measure the distribution

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of this material when injected iv, and [^{32}P]phosphonated polyethylene and methylene [^{32}P]diphosphonate were used to determine rate of uptake from the gut, tissue distribution of the ingested materials being compared with that following injection. Secondly several phosphonated polymers were tested for their effects on caries formation in rats and the experimental animals ingested these materials at low concentrations in their food for 16 wk (Anbar *et al.* 1973). A number of possible toxicological effects were examined at the same time. Lastly, acute toxicity tests were carried out in mice and rats to determine the lethal dose of a number of phosphonated polymers.

EXPERIMENTAL

Materials

Phosphonated polyethylene was prepared by the method of Schroeder & Sopchak (1960) with small modifications described by Anbar *et al.* (1973). The materials tested were a phosphonated polyethylene of low molecular weight (mol wt 6000; 14% P) and a phosphonated polyethylene of high molecular weight (mol wt 50,000; 16% P). The preparation of polyvinylbenzyl phosphonate and polyvinyl phosphonate has been described before (Anbar *et al.* 1973).

Methylene diphosphonate was prepared from the reaction of di-iodomethane with triethyl phosphite to give the diphosphonic tetraethyl ester. This was then hydrolysed in HCl to yield the methylene diphosphonic acid, which was converted to the sodium salt and evaporated to dryness.

The ^{32}P-labelled polyphosphonate (mol wt approximately 3500) was prepared by the method of Leaffer & Anbar (1971). Methylene diphosphonate with a single ^{32}P label was synthesized by condensing triethyl [^{32}P]phosphite with diethyl iodomethyl phosphonate. Fractionation of the products of reaction followed by acid hydrolysis of the appropriate fraction yielded a product with radioactivity occurring only on the desired molecule:

$$^{32}P(OEt)_3 + ICH_2P(OEt)_2 \rightarrow H_2C[^{32}P(OEt)_2]_2.$$

Tissue distribution studies

Twelve male Sprague–Dawley rats (body weight 150 g) were injected via the tail vein with 0·50 ml [^{32}P]polyphosphonate (mol wt about 6000) in saline solution (0·67 mg/ml). Pairs of rats were killed at 2, 4 and 8 hr for tissue distribution studies. The other six rats were housed in individual metabolism cages equipped with urine–faeces separators and were killed in pairs at 24, 48 and 72 hr. The distribution of radioactive polyethylene polyphosphonates of high and low molecular weight was also compared with that of labelled methylene diphosphonate and inorganic phosphate, all administered by iv injection. The doses injected were about 0·5 μCi/rat. The specific activity of the organic phosphonates was about 1 mCi/g, whereas the inorganic phosphate was administered with carrier at a specific activity of 10 mCi/g. The distribution of the radioactivity in the liver, kidney and tibia was measured 8 hr after the injection.

To examine whether polyethylene polyphosphonates were transported across the gut, two lots of labelled polyphosphonates were synthesized, from polyethylenes of molecular weight 3500 and 30,000. In parallel, labelled methylene diphosphonate and phosphate ions were used for comparison. These materials were given by oral injection to 6-wk-old rats (body weight 190–200 g). Each material was given orally to six rats in four daily doses

providing 0·5 μCi radiophosphorus. On day 6, all 24 rats were sacrificed and dissected, and samples of whole blood, serum, liver, kidney and tibia were taken for phosphorus counting.

Radioactivity in the tissues was determined by transferring them into counting polystyrene test tubes (15 × 150 mm), weighing them in the tubes and counting them in a lead-shielded well counter. The detector was a sodium iodide (thallium-activated) scintillation crystal lined with 0·01 in. aluminium, integral with a photomultiplier tube operated at 1400 v. A liquid standard showed the scintillation counter unit to be approximately 12% efficient for ^{32}P betas (β max 1·7 Mev) with the discriminator set for optimal S/BG ratio. An Atomic Instrument Company counter, Model 1070A, with a Cyclotron Specialty clock control completed the counting equipment.

Subacute toxicity study

The animals used in the subacute study were Osborne–Mendel caries-susceptible (OMS) rats bred in our laboratories. The cariogenic diet, purchased from Nutritional Biochemicals, Inc., Cleveland, Ohio, consisted of 61% sucrose, 35% powdered whole milk, 3% alfalfa meal and 1% sodium chloride. The experimental diets were prepared weekly and kept in drums over Drierite (W. A. Hammond Co., Yellow Springs, Ohio). De-ionized water was given *ad lib.* to all experimental groups.

Each experimental group consisted of six male and six female rats housed in groups of three according to sex, and maintained on a regular stock ration for 10 days after weaning. The rats were then randomly distributed into the test groups and maintained on the cariogenic diet, to which the test materials (polyethylene polyphosphonate of high and low molecular weight, polyvinylbenzyl phosphonate and methylene diphosphonate) were added at a level of 1000 ppm throughout the 115-day experimental period. The same animals were treated topically at daily intervals with 0·2 g Orabase (a carboxymethylcellulose preparation, Orahesive, in a liquid paraffin–polyethylene base, from Squibb) containing 2% of the test material.

In addition to an evaluation of anticariogenic activity, observations of general health were made daily, feed consumption and body weights were recorded weekly, the mortality rate was recorded and organ-to-body weight ratios for liver, kidney, spleen, heart, testis and brain were determined on all surviving animals.

Acute toxicity experiments

Young adult male Sprague–Dawley-derived rats (body weight 88–120 g), and Swiss Webster mice (18–26 g) were used. After the rats and mice were fasted overnight, large single doses of the compounds were administered intragastrically as aqueous solutions by means of a ball-point needle and syringe. All animals were observed for 2 wk after treatment and each animal was autopsied and examined for evidence of gross pathology at the end of the observation period.

RESULTS

Tissue distribution of injected ^{32}P-labelled polyethylene polyphosphonate

The distribution of the iv administered ^{32}P-labelled polyethylene polyphosphonate in the tissues of the rats is shown in Table 1. After an initial rapid drop in activity, the serum showed a clearance half-life of about 10 hr. The liver radioactivity built quickly to a

Table 1. *Distribution of radioactivity in the tissues of rats injected with ^{32}P-labelled polyethylene polyphosphonate*

Time after injection (hr)	Radioactivity (% of injected dose/g wet tissue)										
	Vertebrae*	Skull*	Leg bones*†	Incisors*	Liver	Lung	Heart	Muscle	Kidney	Brain	Serum
2	1·6	0·65	2·5	0·3	10·5	1·1	0·7	0·1	3·7	0·10	6·0
4	1·2	0·65	1·9	0·5	6·1	0·4	0·4	0·3	4·4	0·02	1·9
8	2·3	1·2	3·6	1·3	14·0	0·8	0·6	0·2	6·7	0·04	1·4
24	2·4	2·0	4·7	2·2	13·6	1·0	0·6	0·2	7·6	0·05	0·4
48	3·4	2·3	6·2	2·7	12·1	0·5	0·2	0·2	8·5	0·06	0·3
72	3·6	2·9	6·7	3·1	14·1	0·8	0·3	0·2	7·2	0·08	0·15

*Bones were ashed prior to counting and the % of dose/g wet tissue was calculated using the average ratios of the weights of the wet tissue to ash, which were 5·38 ± 0·55 for the vertebrae, 2·77 ± 0·21 for the skull, 3·44 ± 0·44 for the tibia and fibula and 2·22 ± 0·13 for the incisor. These values are the means ± SD.

†Tibia and fibula.

Values are means for two animals.

high and constant level, while the kidney level rose somewhat more slowly and then reached a high constant level. Muscle and brain took up very little of the activity. The heart and lungs accumulated a level comparable to that circulating in the serum. Of the bones, the leg bones and vertebrae showed the most activity, whereas the dense bones of the skull and teeth took up activity more slowly.

The percentages of polyethylene polyphosphonate excreted on days 1, 2 and 3 respectively were 7·7 ± 0·9 (6), 4·0 ± 0·9 (4) and 1·8 ± 0·5 (2) in the faeces and 17·0 ± 8·1 (6), 4·4 ± 1·0 (4) and 3·8 ± 1·2 (2) in the urine, the figures in parentheses indicating the numbers of rats from which the given means were derived. Examination of these excretion data reveals that about 25% of the injected dose was excreted on day 1, after which retained material was excreted at a rate of about 10%/day. Faecal levels decreased with a half-life of 20 hr in the first 3 days. The urine data did not follow a logarithmic decay, probably because of the multi-compartmental uptake of polyethylene polyphosphonate.

Table 2 shows the distribution of radioactivity in the liver, kidney and tibia following administration of ^{32}P-labelled polyethylene polyphosphonate of high or low molecular weight, methylene diphosphonate or inorganic phosphate in the iv study run parallel to the oral-intake experiment subsequently discussed.

Table 2. *Distribution of radioactivity in the tissues of rats injected with ^{32}P-labelled phosphonates and phosphate ions*

Test material	Injected dose (μCi)	Radioactivity (% of injected dose/g wet tissue)		
		Liver	Kidney	Tibia
Polyphosphonate LMW	0·44	8·0 ± 1·1	4·4 ± 0·40	4·0 ± 0·36
HMW	0·55	1·0 ± 0·33	1·8 ± 0·33	1·9 ± 0·23
Methylene diphosphonate	0·34	0·035 ± 0·007	0·031 ± 0·004	2·3 ± 0·28
Phosphate	0·44	0·059 ± 0·012	0·064 ± 0·010	2·0 ± 0·26

LMW = Material of low molecular weight (6000)
HMW = Material of high molecular weight (50,000)
Values are means for groups of six animals ± SD.

These data show a relatively higher uptake of the low-molecular-weight material by the liver and a relatively higher uptake of the high-molecular-weight compound by the kidney. The inorganic phosphate and methylene diphosphate are not taken up extensively by the liver or kidney, but they evidently enter into the phosphate pool and are effectively bound in the bone. The ratio tibia/liver is 0·5–2 for the polyphosphonates, compared with a ratio of 66 for the diphosphonate and 33 for the phosphate. It should be noted, however, that the uptake in bone expressed in terms of the percentage of the injected dose/g tissue is comparable for the polyphosphonates, diphosphonate and phosphate ions.

Tissue distribution of ingested labelled polyethylene polyphosphonates

The rats used for the absorption and distribution studies on orally administered materials showed normal growth during the test, reaching 235–245 g on day 6. The tissue distribution data given in Table 3 show little uptake of radioactive phosphorus through the gut. The only organ that showed a significant uptake was bone, where less than 0·05% of the ingested material was found. This small amount was due primarily to a trace impurity of

inorganic phosphate in the polyphosphonate or to a relaese of phosphate by the intestinal flora, rather than to transport of polyethylene polyphosphonate across the intestinal membrane. The ratio of uptake in the tibia to that in the liver was comparable for the polyethylene polyphosphonates of low and high molecular weight, methylene diphosphonate and phosphate, namely, 31, 28, 39 and 33 respectively. Comparison of these findings with the results of experiments in which the distribution of the same compounds was examined after iv injection, shows that the distribution of the radioactivity of the ingested polyphosphonates resembles that of the phosphate ions given by either route. If the polyphosphonate reached the circulation, we would expect a tibia/liver ratio of less than unity (see Tables 1 & 2). There seems to be an insignificant difference in the extent of uptake of materials of high and low molecular weight, which again indicates that the activity found is due to inorganic phosphate.

Table 3. *Distribution of radioactivity in the tissues of rats fed ^{32}P-labelled phosphonates and phosphate ions*

	Radioactivity (% of ingested dose/g wet tissue × 10^4)				
Test material	Whole blood	Serum	Liver	Kidney	Tibia
Polyphosphonate LMW	1·7 ± 0·8	1·1 ± 0·7	0·35 ± 0·25	1·6 ± 1·2	10·9 ± 3·5
HMW	1·4 ± 5·0	1·2 ± 5·0	0·4 ± 0·15	1·05 ± 0·6	11·3 ± 4
Methylene diphosphonate	23 ± 10	—	7 ± 2	8 ± 1	272 ± 40
Phosphate	310 ± 56	—	70 ± 2	66 ± 4	2330 ± 240

LMW = Material of low molecular weight (6000)
HMW = Material of high molecular weight (50,000)
Values are means for groups of six animals ± SD.

Consequently, the apparent total intake of polyphosphonate through the gut, which amounts to less than 0·05% (based on the uptake by bone), is over 95% due to phosphate ions. This puts the upper limit of uptake of polyphosphonate through the gut of rats at $2{\cdot}5 \times 10^{-5}$% of the ingested dose.

Subacute "low-dose" ingestion experiment

In this "low-dose" experiment, in which rats were given diets containing 1000 ppm of different polyphosphonates, doses of the order of 50 mg/kg/day were ingested for a total of 115 days. The effect of treatment on body weight and daily food intake is presented in Table 4.

In Experiment 2, one male rat receiving polyethylene polyphosphonate of low molecular weight was cannibalized on day 73, and a second male rat receiving the high-molecular-weight material was found dead on day 89. The cause of death in the two rats receiving the test polyphosphonates was not determined but did not appear to be drug-related. No obvious effects attributable to the test polyphosphonates were noted in the appearance and behaviour of the rats during the 115-day test period, nor was there any adverse effect on body weight and daily food intake (Table 4). Organ-to-body weight ratios of male and female rats sacrificed at the end of the 115-day experimental period are presented in Table 5. Statistically significant increases in relative liver weight were observed in rats given methylene diphosphonate. No other effect of this sort was observed.

Table 4. *Effect of polyphosphonates administered both in the diet and topically on body weights and food intake in rats*

Experiment no.	Experimental group*	No. of rats/group at day 0†	No. of rats/group at day 115	Mean body weight (g ± SD) at day 0	Mean body weight (g ± SD) at day 115	Mean food intake over 0–115 days (g/rat/day ± SD)
1	Control	12	12	82·2 ± 2·6	240·3 ± 10·9	12·3 ± 0·5
	PP (LMW)	12	12	82·5 ± 3·5	246·7 ± 9·6	12·2 ± 0·2
	PVBP	12	12	84·7 ± 4·5	250·5 ± 12·9	12·4 ± 0·2
2	Control	12	12	60·5 ± 2·5	211·3 ± 9·0	10·2 ± 0·4
	PP (LMW)	12	11	69·5 ± 2·7	242·6 ± 10·9	11·2 ± 0·3
	PP (HMW)	12	11	67·1 ± 4·1	234·7 ± 12·6	11·7 ± 0·3
	PVP	12	12	70·6 ± 3·3	226·0 ± 14·9	11·0 ± 0·2
	MD	12	12	71·4 ± 3·7	225·0 ± 5·9	10·6 ± 0·5

PP = Polyethylene polyphosphonate LMW = Low molecular weight HMW = High molecular weight
PVBP = Polyvinylbenzyl phosphonate PVP = Polyvinyl phosphonate MD = Methylene diphosphonate
*All test polyphosphonates were administered at a standard level of 1000 ppm in the diet and at a level of 2% in Orabase administered topically.
†Each group consisted of six males and six females.

Table 5. *Effect of polyphosphonates administered both in the diet and topically on organ/body weight ratios in rats*

Experiment no.	Experimental group†	No. of rats/group	Relative organ weight (g/100 g body weight)					
			Liver	Kidney	Spleen	Heart	Testis	Brain
			Males					
1	Control	6	4·39	0·78	0·16	0·47	1·11	0·74
	PP (LMW)	6	4·50	0·83	0·26	0·53	1·01	0·77
	PVBP	6	4·41	0·82	0·21	0·57	1·25	0·73
			Females					
	Control	6	4·56	0·93	0·23	0·56	—	0·93
	PP (LMW)	6	4·63	0·89	0·31	0·63	—	0·88
	PVBP	6	4·39	0·90	0·26	0·62	—	0·97
			Males					
2	Control	6	4·32	0·75	0·26	0·47	1·21	0·81
	PP (LMW)	5	4·45	0·79	0·19	0·51	1·11	0·72
	PP (HMW)	5	4·70	0·75	0·23	0·52	1·14	0·74
	PVP	6	4·01	0·76	0·20	0·48	1·24	0·78
	MD	6	4·83*	0·72	0·24	0·48	1·22	0·79
			Females					
	Control	6	4·28	0·76	0·30	0·56	—	0·92
	PP (LMW)	6	4·11	0·77	0·30	0·55	—	0·84
	PP (HMW)	6	4·34	0·83	0·32	0·61	—	0·90
	PVP	6	4·01	0·79	0·25	0·55	—	0·87
	MD	6	4·70*	0·80	0·25	0·53	—	0·86

PP = Polyethylene polyphosphonate LMW = Low molecular weight HMW = High molecular weight
PVBP = Polyvinylbenzyl phosphonate PVP = Polyvinyl phosphonate MD = Methylene diphosphonate

†All test polyphosphonates were administered at a standard level of 1000 ppm in the diet and at a level of 2% in Orabase administered topically. Controls were treated only with topically applied Orabase containing no test material.

Values are means for the numbers of rats indicated and those marked with an asterisk differ significantly from that of the corresponding non-medicated Orabase control: $*P < 0{\cdot}05$.

At autopsy, gross pathological findings were randomly distributed throughout all test and control groups. The conditions generally found were a slight-to-moderate swelling of the liver and varying amounts of atelectasis of the lung. None of the gross findings were considered to be attributable to treatment.

Acute toxicity

The results of administering orally a large single dose of polyethylene polyphosphonate or polyvinyl phosphonate to mice and rats show that these materials have a very low degree of toxicity.

Mice given low-molecular-weight polyethylene polyphosphonate in a dose of 1·25, 2·50, 5·0 or 10·0 g/kg exhibited no abnormalities throughout the experiment. Since there were no abnormal responses in the mice, a single treatment level of 10 g/kg was selected for the rat study. One rat was found dead 24 hr after treatment; there was mild-to-moderate diarrhoea in the other treated animals for approximately 24 hr. No other abnormalities were evident. These experiments established the acute oral toxicity of this polyphosphonate expressed in terms of the LD_{50} as >10 g/kg in rats and mice.

Mice given polyethylene polyphosphonate of high molecular weight in a dose of 1·25, 2·50, 5·0 or 10·0 g/kg exhibited no abnormalities. Thus, a single treatment level of 10 g/kg was again used for the rat study. There was mild diarrhoea for approximately 12 hr in the treated rats, but no other abnormalities were evident. These data show the acute oral toxicity (LD_{50}) of this high-molecular-weight material to rats and mice to be >10 g/kg.

Rats and mice given 10 g polyvinyl phosphonate/kg exhibited no abnormal responses. The acute oral toxicity (LD_{50}) of polyvinyl phosphonate to rats and mice is >10 g/kg.

DISCUSSION

From the toxicological findings, it may be concluded that polymeric polyphosphonates are non-toxic when administered orally. This finding is not surprising in view of the extremely low absorption of these polyelectrolytes from the gut. In contrast, oligophosphonates, exemplified by methylene diphosphonate, are absorbed from the gut and may thus affect bone metabolism and possibly other organs as well. (Liver enlargement was observed in the experiment involving prolonged intake of a dose of this compound of the order of 50 mg/kg/day.)

Polymeric polyphosphonates may be used as a dentifrice additive for caries and calculus prevention. Our findings permit a rough estimate to be made of the toxicological hazard involved in such an application. Let us assume that the concentration of a polymeric polyphosphonate in the dentifrice is 2%, that the daily consumption of dentifrice is 1 g and that 10% of this amount is ingested. This would mean an ingested dose of 2 mg/day or about 30 μg/kg/day. This dose is 1000 times less than the daily amount ingested in our "low-dose" experiments, and about six orders of magnitude lower than the dose used in our acute experiment, which also failed to produce toxic effects. From the distribution and uptake experiments, it may be estimated that the upper limit of systemic uptake of polymeric polyphosphonates used in a dentifrice is of the order of 10^{-11} g/kg/day, which is more than ten orders of magnitude lower than the doses found necessary to affect bone metabolism (Anbar *et al.* 1971a,b).

Although this study is considered to be merely a preliminary toxicological examination of a new family of compounds, the results strongly suggest that if polymeric polyphosphonates are found to be effective dental prophylactic agents, no serious toxicological hazard should be encountered.

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Schicksal und Toxizität oral angewendeter Polyäthylenpolyphosphonate

Zusammanfassung—Die möglichen toxischen Wirkungen organischer polymerer Polyphosphonate, die zur Prophylaxe von Zahnkaries und Nephrolithiasis verwendet werden könnten, wurden untersucht. Die Resorption dieser Polymere aus dem Darm, die unter Verwendung von ^{32}P-markierten Materialien geprüft wurde, ist äusserst gering—wienger als $2{,}5 \times 10^{-5}\%$ der aufgenommenen Dosis. Untersuchungen der subakuten Toxizität an Ratten, an die Polyäthylenpolyphosphonat mit einer Dosis im Futter verabreicht wurde, die einer Aufnahme von etwa 50 mg/kg/Tag entsprach, und der akuten Toxizität an Mäusen und Ratten, die eine einzelne orale Dosis bis 10 g/kg erhielten, lieferten keinen Beweis irgendwelcher signifikanter nachteiliger Wirkungen. Die beobachtete niedrige Toxizität wird mit der extrem langsamen Aufnahme dieser Verbindungen in den Körper erklärt.

Destin et toxicité des polyphosphonates de polyéthylène administrés par boie orale

Résumé—On a examiné si des polyphosphonates, polymères organiques susceptibles d'être employés en prophylaxie des caries et calculs dentaires, peuvent avoir des effets toxiques. L'absorption intestinale de ces polymères, marqués au ^{32}P, est extrêmement faible, puisque inférieure à $2{,}5 \times 10^{-5}\%$ de la dose ingérée. La toxicité subaiguë a été étudiée sur des rats, dont le régime comportait une dose de polyphosphonate de polyéthylène assurant une ingestion d'environ 50 mg/kg/jour, et la toxicité aiguë sur des rats et des souris qui recevaient une dose orale unique s'élevant jusqu'à 10 g/kg. Ni l'une ni l'autre épreuve n'ont révélé d'effets nocifs significatifs. La faible toxicité constatée s'explique par l'absorption extrêmement faible de ces produits par l'organisme.

Fd Cosmet. Toxicol. Vol. 11, pp. 1011–1081. Pergamon Press 1973. Printed in Great Britain

MONOGRAPHS

Monographs on Fragrance Raw Materials*

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BENZYL ALCOHOL

Synonym: Phenyl carbinol.
Structure: $C_6H_5 \cdot CH_2OH$.
Description and physical properties: Food Chemicals Codex (1972).
Occurrence: Found in jasmine, hyacinth, ylang-ylang oils and at least two dozen other essential oils (Gildemeister & Hoffman, 1962).
Preparation: By the action of alkalis on benzyl chloride (Bedoukian, 1967).
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to about 250,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·01	0·05	0·40
Maximum	0·15	0·025	0·15	1·0

Status

Benzyl 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 (1970) listed benzyl alcohol, giving an ADI of 5 mg/kg. The *Food Chemicals Codex* (1972) and the National Formulary (1970) have monographs on benzyl alcohol and another extensive monograph has been provided by Browning (1965).

Biological data

Acute toxicity. The single-dose oral LD_{50}s in rats and rabbits were reported as 2·08 and 1·94 g/kg, respectively (Graham & Kuizenga, 1945). The acute oral LD_{50} was reported as 1230 mg/kg in rats (Bär & Griepentrog, 1967) and as 1580 mg/kg in the mouse (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} in guinea-pigs was reported to be $< 5{\cdot}0$ ml/kg (Jones, 1967). The approximate LD_{50} ip was reported as 400–800 mg/kg in rats and guinea-pigs (Treon, 1963).

*Previous sets of these monographs were published in *Food and Cosmetics Toxicology* (1973, **11**, 95, 477 & 855).

Irritation. The undiluted material applied to the depilated skin of guinea-pigs for a period of 24 hr caused moderately strong primary irritation (Treon, 1963).

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

Metabolism. Esters of benzyl alcohol (acetate, benzoate, cinnamate and hydrocinnamate) are rapidly hydrolysed *in vivo* to benzyl alcohol, which is then oxidized (Williams, 1959). The animal (and human) organism readily oxidizes benzyl alcohol to benzoic acid, which after conjugation with glycine is rapidly eliminated as hippuric acid in the urine (Treon, 1963).

Additional published data

Slow iv injection of 1 ml 0·9% benzyl alcohol/kg into unanaesthetized and anaesthetized dogs and anaesthetized monkeys failed to alter the blood, heart rate, respiration or ECG pattern, and produced no changes in the haematological picture. Intracarotid and intrarenal injection of the same dose into anaesthetized dogs did not affect either these parameters or the EEG tracing. The lethal iv dose of benzyl alcohol in anaesthetized dogs was 830–1060 mg/kg and the LD_{50} in rats was 314 mg/kg, while in mice 480 mg/kg was uniformly fatal when injected rapidly. In rats, 94% benzyl alcohol was 23 times more toxic iv than 95% ethanol, and again in mice, in which the LD_{50} of benzyl alcohol was below 480 mg/kg, the LD_{50} for 95% ethanol was 1460 mg/kg (Kimura, Darby, Krause & Brondyk, 1971).

Two methylprednisolone sodium succinate formulations with different preservatives (benzyl alcohol and parabens) and a placebo were administered iv in single doses of 2·0 g to 24 subjects. Both formulations were well tolerated and no important drug-related side effects were encountered. No clinically significant changes in the vital signs, electrocardiograms, electroencephalograms or laboratory parameters were noted. All expected corticosteroid-induced changes were reversible. The higher antibacterial activity of benzyl alcohol shown in the challenge tests plus comparable tolerance to parabens favour the use of benzyl alcohol as a preservative (Novak, Stubbs, Sanborn & Eustice, 1972).

Cross-sensitizations to benzyl alcohol have been reported in subjects sensitized to Peru balsam (Hjorth, 1961).

Benzyl alcohol vapours can penetrate the intact skin, and vapour concentrations of approximately 100 ppm can cause systemic effects and deaths in laboratory animals. A tentative vapour exposure level of 1 ppm may prove acceptable for workroom atmospheres. Due to its lachrymatory effects, exposures much above 1 ppm would probably not be tolerated for any prolonged period (Jones, 1967).

Hypersensitivity to phenyl carbinol preservative in Vitamin B_{12} (for injection) has been reported (Lagerholm, 1958).

Benzyl alcohol exerts a narcotic action but is of low toxicity. High exposure can cause a decrease in blood pressure, a depressant effect on the system and death through respiratory paralysis (*Handbook of Organic Industrial Solvents*, 1961).

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BENZYL BENZOATE

Synonym: Benzoic acid benzyl ester.
Structure: $C_6H_5 \cdot CH_2 \cdot CO_2 \cdot C_6H_5$.
Description and physical properties: Food Chemicals Codex (1972).
Occurrence: Found in Peru and Tolu balsams, in ylang-ylang and in about twenty other essential oils (Gildemeister & Hoffman, 1966).
Preparation: By the interaction of sodium benzoate and benzyl chloride (Bedoukian, 1967).
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to about 500,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·005	0·10	0·40
Maximum	0·10	0·010	0·25	3·00

Status

Benzyl 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 (1970) listed benzyl benzoate, giving an ADI of 5 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on benzyl benzoate.

Biological data

Acute toxicity. The single-dose oral LD_{50}s in rats and rabbits were reported as 2·8 and 1·68 g/kg, respectively, while in the cat the LD_{50} was reported as 2·24 g/kg and in the dog as >22·44 g/kg (Graham & Kuizenga, 1945). The acute dermal LD_{50} in the rabbit was reported as 4 ml/kg (Fassett, 1963).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 30% concentration in petrolatum and produced no sensitization reactions (Kligman, 1970).

Metabolism. Benzyl benzoate, a relatively non-toxic liquid widely used for the treatment of scabies, is converted into benzoic acid *in vivo* (Williams, 1959).

Additional published data

Cross-sensitization reactions to benzyl benzoate have been reported in subjects sensitized to Peru balsam (Hjorth, 1961).

Benzyl benzoate is a primary skin irritant (Schwartz, Tulipan & Birmingham, 1957), but used as a 20% emulsion in the treatment of scabies in 1000 persons it produced no dermatitis (Graham, 1943). Four cases of dermatitis have been attributed to benzyl benzoate by Dougherty (1945). Benzyl benzoate is an effective treatment against scabies and does not often cause dermatitis (Mellanby, 1963).

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BENZYL CINNAMATE

Synonyms: Benzyl-β-phenylacrylate; cinnamein.
Structure: $C_6H_5 \cdot CH_2 \cdot CO_2 \cdot CH{:}CH \cdot C_6H_5$.
Description and physical properties: EOA Spec. no. 124.
Occurrence: Found in balsams of Peru, tolu, styrax, copaiba and others.
Preparation: By the esterification of benzyl alcohol and cinnamic acid, or by the interaction of benzyl chloride and sodium cinnamate.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·005	0·05	0·2
Maximum	0·10	0·02	0·2	0·8

Analytical data: Gas chromatogram, RIFM no. 71–35; infra-red curve, RIFM no. 71–35.

Status

Benzyl cinnamate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed benzyl cinnamate, giving an ADI of 1·25 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on benzyl cinnamate.

Biological data

Acute toxicity. The acute oral LD_{50} was found to be 3·28 g/kg (2·62–4·10 g/kg) in rats (Levenstein, 1972a) and 3·76 g/kg int he guinea-pig (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} was reported to be > 3·0 g/kg in the rabbit (Levenstein, 1972).

Chronic toxicity: In a feeding study, 10,000 ppm fed to rats in the diet for 19 weeks produced no macroscopic effects (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

Irritation. Benzyl cinnamate applied full strength to intact or abraded rabbit skin was mildly irritating (Levenstein, 1972b). Tested in a concentration of 8% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

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 (Kligman, 1972).

Metabolism. See monograph on Benzyl alcohol (p. 1011).

References

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Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2142. *Fd Technol., Champaign* **19**(2), part 2, 155.

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BENZYL FORMATE

Synonym: Formic acid benzyl ester.
Structure: $C_6H_5 \cdot CH_2 \cdot CO_2 \cdot H$.
Description and physical properties: Givaudan Index (1961).
Occurrence: Apparently has not been reported to occur in nature.
Preparation: From formic acid and benzyl alcohol (*Merck Index*, 1968).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·010	0·002	0·005	0·05
Maximum	0·020	0·008	0·020	0·95

Analytical data: Gas chromatogram, RIFM no. 71–36; infra-red curve, RIFM no. 71–36.

Status

Benzyl formate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed benzyl formate, giving an ADI of 5 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was found to be 1·7 ml/kg (1·4–2·1 ml/kg) (Shelanski & Moldovan, 1971). The acute dermal LD_{50} in rabbits was found to be 2·0 ml/kg (1·3–3·0 ml/kg) (Shelanski & Moldovan, 1971).

Irritation. Benzyl formate tested at a 10% concentration in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a 10% concentration in petrolatum and produced no sensitization reactions (Kligman, 1971). Benzyl formate at full strength produced no reactions in a single patch test on 20 humans (Peterson & Hall, 1946).

References

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BENZYLIDENE ACETONE

Synonyms: 4-Phenyl-3-buten-2-one; benzalacetone.
Structure: $C_6H_5 \cdot CH:CH \cdot CO \cdot CH_3$.
Description and physical properties: Givaudan Index (1961).
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By condensing acetone and benzaldehyde by means of aqueous alkali (*Merck Index*, 1968).
Uses: In public use since the 1920s.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·005	0·005
Maximum	0·01	0·005	0·01	0·05

Analytical data: Gas chromatogram, RIFM no. 71–41; infra-red curve, RIFM no. 71–41.

Status

Benzylidene acetone was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164).

Biological data

Acute toxicity. The acute oral LD_{50} was reported as >5 g/kg in the rat (Levenstein, 1972a). The acute dermal LD_{50} was reported as >3 g/kg in the rabbit (Levenstein, 1972b).

Irritation. Benzylidene acetone applied full strength to intact or abraded rabbit skin was mildly irritating (Levenstein, 1972b). Tested at a 2% concentration in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a 2% concentration in petrolatum and produced sensitization reactions in 12 out of 25 subjects (Kligman, 1972).

Additional published data

Benzylidene acetone is listed as a strongly irritant perfume material (Thomssen, 1947), and has been cited many times in the literature as a skin irritant.

References

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BENZYL ISOBUTYRATE

Synonym: Benzyl 2-methyl propionate.
Structure: $C_6H_5 \cdot CH_2 \cdot CO_2 \cdot CH(CH_3) \cdot CH_3$.
Description and physical properties: EOA Spec. no. 267.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By esterification of benzyl alcohol with isobutyric acid.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfumes*
Usual	0·02	0·004	0·021	0·041
Maximum	0·06	0·010	0·075	0·41

Analytical data: Gas chromatogram, RIFM no. 71–37; infra-red curve, RIFM no. 71–37.

Status

Benzyl isobutyrate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included benzyl isobutyrate in the list of admissible artificial flavouring substances at a level of 10 ppm.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was found to be 2850 mg/kg (2111–3847 mg/kg) (Owen, 1971a). The acute dermal LD_{50} was reported to be > 5 ml/kg in the rabbit (Owen, 1971b).

Irritation. Benzyl isobutyrate tested at a concentration of 4% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1971).

References

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Owen, G. (1971b). Report to RIFM, 1 July.

BENZYL ISOEUGENOL

Synonyms: Isoeugenol benzyl ether; benzyl 2-methoxy-4-propenylphenyl ether.
Structure: $C_6H_5 \cdot CH_2O \cdot C_6H_3(OCH_3) \cdot CH:CH \cdot CH_3$.
Description and physical properties: EOA Spec. no. 237.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By benzylation of isoeugenol.
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	0·005	0·02
Maximum	0·10	0·015	0·05	0·5

Analytical data: Gas chromatogram, RIFM no. 71–38; infra-red curve, RIFM no. 71–38.

Status

Benzyl isoeugenol is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed benzyl isoeugenol, giving an ADI of 5 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 4·9 g/kg (4·71–5·1 g/kg) (Levenstein, 1972a). The acute dermal LD_{50} was reported as > 3 g/kg in the rabbit (Levenstein, 1972b).

Irritation: Benzyl isoeugenol applied full strength to intact or abraded rabbit skin was mildly irritating (Levenstein, 1972b). Tested at a concentration of 5% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a 5% concentration in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 554, p. 77. Strasbourg.
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Levenstein, I. (1972a). Report to RIFM, 13 March.
Levenstein, I. (1972b). Report to RIFM, 7 April.

BENZYL PHENYLACETATE

Synonym: Benzyl α-toluate.
Structure: $C_6H_5 \cdot CH_2 \cdot CO_2 \cdot CH_2 \cdot C_6H_5$.
Description and physical properties: Food Chemicals Codex (1972).
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By direct esterification of benzyl alcohol with phenylacetic acid under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·010	0·07
Maximum	0·08	0·010	0·080	0·2

Analytical data: Gas chromatogram, RIFM no. 71–39; infra-red curve, RIFM no. 71–39.

Status

Benzyl phenylacetate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included benzyl phenylacetate in the list of admissible artificial flavouring substances at a level of 5 ppm. The *Food Chemicals Codex* (1972) has a monograph on benzyl phenylacetate.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as >5000 mg/kg in the rat (Owen, 1971a). The acute dermal LD_{50} was reported as >10 ml/kg in the rabbit (Owen, 1971b).

Irritation. Benzyl phenylacetate was tested at a concentration of 2% in petrolatum and produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a 2% concentration in petrolatum and produced no sensitization reaction (Kligman, 1971). Benzyl phenylacetate has not been reported to cause irritation or sensitivity (Caryl & Ericks, 1939).

References

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Owen, G. (1971b). Report to RIFM, 1 July.

BENZYL SALICYLATE

Synonym: Benzyl *o*-hydroxybenzoate.
Structure: $HO \cdot C_6H_4 \cdot CO_2 \cdot CH_2 \cdot C_6H_5$.
Description and physical properties: EOA Spec. no. 93.
Occurrence: Found in about a dozen essential oils (Gildemeister & Hoffman, 1966).
Preparation: By the interaction of sodium salicylate with benzyl chloride (Bedoukian, 1967)
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to about 300,000 lb/yr.

	Soap	*Detergent*	*Creams, lotions*	*Perfpme*
Usual	0·05	0·005	0·05	0·20
Maximum	0·1	0·01	0·10	3·00

Analytical data: Gas chromatogram, RIFM no. 2883 (HKC); infra-red curve, RIFM no. 2883 (HKC).

Status

Benzyl salicylate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included benzyl salicylate in the list of admissible artificial flavouring substances at a level of 2 ppm. The *Food Chemicals Codex* (1972) has a monograph on benzyl salicylate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 2227 mg/kg (1925–2580 mg/kg) (Fogleman, 1970a). The acute dermal LD_{50} in rabbits was reported as 14,150 mg/kg (13,860–14,560 mg/kg) (Fogleman, 1970b).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 30% in petrolatum and produced no sensitization reactions (Kligman 1970).

Additional published data

Benzyl salicylate has been suggested as a causative agent in patients with dermatitis produced by Peru balsam (Hjorth, 1961). Hypersensitivity or excessive use may cause skin to blister, leading to an increase in pigmentation (Sulzberger & Wolf, 1942). Benzyl salicylate was reported to cause severe pruritus in six of 15 patients who applied it in a trioxsalen lotion. Reactivity to benzyl salicylate was enhanced by the phototoxic effects of methoxsalen. Only one of 14 control patients reacted to the benzyl salicylate (Kahn, 1971).

Benzyl salicylate was studied *in vitro* and *in vivo* in a group of normal subjects and in patients suffering from polymorphic light eruption, solar urticaria, porphyria or xeroderma pigmentosum. It was found that a preparation with an apparently adequate protection factor in normal subjects not infrequently failed to give comparable protection in patients with abnormal skin responses either on phototesting or during clinical use. A combined chemical and physical light-protective preparation was formulated and shown to be more effective (MacLeod & Frain-bell, 1971).

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BERGAMOT OIL EXPRESSED

Description and physical properties: EOA Spec. no. 256.
Occurrence: Found in the fruit of *Citrus bergamia* Risso et Poiteau (Fam. Rutaceae).
Preparation: By cold expression from the peel of the fresh fruit of *Citrus bergamia* Risso et Poiteau (Fam. Rutaceae).
Uses: In public use since the early 1860s. Use in fragrances in the USA amounts to approximately 300,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	—	0·05	0·2
Maximum	0·12	—	0·25	3·00

Status

Bergamot oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included bergamot oil (*Citrus bergamia*) in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on bergamot oil.

Biological data

Acute toxicity. The acute oral LD_{50} (on the rectified oil) was reported as > 10 g/kg in the rat (Fogleman, 1970a). The acute dermal LD_{50} (on the rectified oil) was reported as > 20 g/kg in the rabbit (Fogleman, 1970b).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 30% in petrolatum and produced no sensitization reactions (Kligman, 1970).

Phototoxicity. Severe phototoxic effects have been reported for bergamot oil expressed, using simulated sunlight on hairless mice, pigs and man (Urbach & Forbes, 1972). Severe phototoxic reactions to bergamot oil, expressed, were induced in man using natural sunlight (Wild, 1971). No phototoxic reaction has been encountered in the absence of a characteristic absorption in the ultraviolet spectrum between 312 and 320 nm. The peak may be present without phototoxic properties but so far the reverse has not been found.

Additional published data

There is an abundant literature on bergamot oil; too extensive to report in its entirety. The following references are considered important examples.

There are several articles in the dermatological literature relating bergamot oil and berloque dermatitis. The photodermatitis has been attributed to the content of 5-methoxypsoralen in the expressed oil. This may be as high as 0·39%.

Various components isolated from bergamot oil have been tested for phototoxic effects on human skin. Results suggest that berloque dermatitis is due to a single active component of bergamot, namely bergapten or 5-methoxypsoralen, and that this must be reduced to 0·001% or lower to obviate the effect. The term "bergapten dermatitis" has been suggested as a more accurate name (Marzulli & Maibach, 1970).

The use of furocoumarins in the curing of skin depigmentation problems has been reported to date back to ancient times (Fitzpatrick & Pathak, 1959). Bergamot oil is used

as an effective, permanent treatment against vitiligo (Kenney, 1971). A method for the reproduction of berloque dermatitis has been developed (Harber, Harris & Leider, 1964).

Synthetic furocoumarins, which have their molecules altered with certain groups that cause a change in their absorption and fluorescent spectra, have decreased biological responses (Pathak, Fellman & Kaufman, 1960). A linear fusion of furan and coumarin rings, as in the psoralen molecule, appears to be essential for the phototoxic response; a non-linear structure like isopsoralen has no phototoxic action (Pathak & Fitzpatrick, 1959). Studies on the various phototoxic activities of different psoralens and the mechanisms of their action have been reported (Caporale, Musajo, Rodighiero & Baccichetti, 1967).

A method for identifying, quantitating and characterizing the coumarins and furocoumarins present in the steam non-volatile portion of bergamot oil has been presented (Cieri, 1968).

One study compared the photoreactivity with DNA of psoralen and its 8-methyl, 8-methoxy, 5-methyl, 5-methoxy, 8-hydroxy and 4',5-dihydro derivatives and isopsoralen. Small quantities of tritium-labelled psoralens were irradiated at 365 nm with aqueous calf-thymus DNA at 22°C. After precipitation and re-solution of the DNA, its radioactivity was measured with a liquid scintillation counter to determine the amount of psoralen derivative linked to the DNA. The irradiation time needed to link 20% of the added psoralen to DNA was taken as the measure of photoreactivity. *In vivo* determinations of the activity of these compounds in producing phototoxicity in the skin of guinea-pigs showed a trend parallel to the photoreactivities determined *in vitro*, both types of activity increasing in the order iso-, 5-methoxy-, unsubstituted, 5-methyl- and 8-methyl psoralen. The 8-hydroxy and 4,5-dihydro derivatives were inactive both *in vivo* and *in vitro* (Rodighiero, Musajo, Dall'Acqua, Marciani, Caporale & Ciavatta, 1969). Other related papers have been contributed by Kuske (1938), Pathak, Daniels & Fitzpatrick (1962), Klaber (1942), Musajo & Rodighiero (1962) and Auerbach & Pearlstein (1971). The hairless mouse is an ideal animal for demonstrating phototoxicity (Gloxhuber, 1970).

Dermatological accidents attributed to tanning agents based on bergamot oil have been reported (Meyer, 1970).

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BERGAMOT OIL RECTIFIED

See monograph on Bergamot Oil Expressed (p. 000).

Preparation: By rectification of bergamot oil expressed, under vacuum, to remove completely the furocoumarins and other related non-volatile residues.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as greater than 10 g/kg in the rat (Fogleman, 1970a). The acute dermal LD_{50} was reported to be >20 g/kg in the rabbit (Fogleman, 1970b).

Irritation. Bergamot oil rectified applied full strength to intact or abraded rabbit skin produced a mild irritation (Fogleman, 1970b).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 30% concentration in petrolatum and produced no sensitization reaction (Kligman, 1970).

Phototoxicity. No phototoxic effects have been reported for bergamot oil rectified (Urbach, 1972). No phototoxic reactions to bergamot oil rectified were induced using natural sunlight (Wild, 1971).

No phototoxic reaction has been encountered in the absence of a characteristic absorption in the ultraviolet spectrum between 312 and 320 nm. The peak may be present without phototoxic properties but so far the reverse has not been found.

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Wild, E. (1971). Report to RIFM, 20 August.

BIRCH TAR OIL

Description and physical properties: EOA Spec. no. 105.
Occurrence: Found in the tar of the bark and wood of *Betula pendula* Roth (fam. Betulaceae).
Preparation: By steam-distillation of the tar obtained by dry distillation of the bark and the wood of *Betula pendula* Roth (fam. Betulaceae).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·002	0·02
Maximum	0·01	0·005	0·01	0·2

Status

Birch tar oil is approved by the FDA for food use (21 CFR 121.1164). The *Food Chemicals Codex* (1972) has a monograph on birch tar oil.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as > 5 g/kg in the rat (Moreno, 1972a). The acute dermal LD_{50} was reported as > 2 g/kg in the rabbit (Moreno, 1972b).

Irritation. Undiluted birch tar oil applied to the backs of hairless mice produced no irritating effects (Urbach & Forbes, 1972). Birch tar oil applied full strength to intact or abraded rabbit skin produced irritation (Moreno, 1972b). Birch tar oil tested at a concentration of 2% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. No phototoxic effects have been reported for birch tar oil (Urbach, 1972)

Additional published data

An occasional individual may be hypersensitive to birch tar oil (Schwartz, 1934).

References

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Urbach, F. & Forbes, P. D. (1972). Report to RIFM, 26 May.

BOIS DE ROSE ACETYLATED

Description and physical properties: A colourless or pale-yellow liquid with a spicy-lavender odour (Arctander, 1960).

Occurrence: The rosewood oil prior to acetylation is obtained by steam-distillation of the wood of *Aniba rosaeodora.*

Preparation: By acetylation of Bois de Rose oil (Arctander, 1960).

Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 30,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·1	0·01	0·1	0·4
Maximum	0·3	0·03	0·3	1·2

Analytical data: Gas chromatogram, RIFM no. 72–10; infra-red curve, RIFM no. 72–10.

Status

Bois de rose acetylated is not listed by the Council of Europe (1970), FEMA, the FDA or the *Food Chemicals Codex* (1972), but the parent substance is. Bois de rose oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included bois de rose oil (*Aniba rosaeodora*), in the list of temporarily admitted flavouring substances. The *Food Chemicals Codex* (1972) has a monograph on bois de rose.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as >5 g/kg in the rat (Keating, 1972). The acute dermal LD_{50} was reported as >5 g/kg in the rabbit (Keating, 1972).

Irritation. Bois de rose acetylated applied undiluted to the backs of hairless mice produced no irritating effects (Urbach, 1973). At a concentration of 12% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 12% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. No phototoxic effects have been reported for bois de rose acetylated (Urbach, 1973).

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laevo-BORNYL ACETATE

Synonym: l-1,7,7,-Trimethyl-2-*endo*-acetyloxy-bicyclo-(2,2,1)-heptane.
Structure: $CH_3 \cdot CO \cdot OC_{10}H_{17}$.
Description and physical properties: EOA Spec. no. 170.
Occurrence: Found in many oils distilled from the leaves of plants of the family Pinaceae as well as in other volatile oils such as Coriander seed and Valerian root oil.
Preparation: By isolation from various pine needle oils by fractional distillation or by acetylation of *l*-borneol.
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 3000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·020	0·05	0·05
Maximum	0·60	0·100	0·30	0·2

Analytical data: Gas chromatogram, RIFM no. 71–43; infra-red curve, RIFM no. 71–43.

Status

l-Bornyl 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 (1970) included *l*-bornyl acetate in the list of admissible artificial flavouring substances at a level of 2 ppm. The *Food Chemicals Codex* (1972) has a monograph on *l*-bornyl acetate.

Biological data

Acute toxicity. An acute oral LD_{50} was reported as >5000 mg/kg in the rat (Owen, 1971a). The acute dermal LD_{50} was reported as >10 ml/kg in the rabbit (Owen, 1971b).

Irritation. l-Bornyl acetate tested at a concentration of 2% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

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

Additional published data

Various tobacco leaf and tobacco smoke components and related synthetic compounds were tested for co-carcinogenic activity on mouse skin by simultaneous and repeated application with benzo[*a*]pyrene. The three corresponding bornyl esters showed no co-carcinogenic activity (Van Duuren, Blazej, Goldschmidt, Katz, Melchionne & Sivak, 1971).

References

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Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2159. *Fd Technol., Champaign* **19**(2), part 2, 155.

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Van Duuren, B. L., Blazej, T., Goldschmidt, B. M., Katz, C., Melchionne, S. & Sivak, A. (1971). Co-carcinogenesis studies on mouse skin and inhibition of tumor induction. *J. natn. Cancer Inst.* **46**, 1039.

BROMSTYROL

Synonym: α-Bromo-β-phenylethylene.
Structure: $C_6H_5 \cdot CH:CHBr$.
Description and physical properties: EOA Spec. no. 150.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By the action of aqueous alkali on cinnamic acid dibromide.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·002	0·001
Maximum	0·02	0·01	0·01	0·04

Analytical data: Gas chromatogram, RIFM no. 71–44; infra-red curve, RIFM no. 71–44.

Status

The Council of Europe (1970) included bromstyrol in the list of temporarily admissible artificial flavouring substances.

Biological data

Acute toxicity. An acute oral LD_{50} in rats was reported as 1·25 ml/kg (0·82–189 ml/kg) (Levenstein, 1972a). The acute dermal LD_{50} was reported as >6 ml/kg in the rabbit (Levenstein, 1972b).

Irritation. Bromstyrol tested at a concentration of 4% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Additional published data

Bromstyrol is one of the strongly irritant perfume materials (Thomssen, 1947). Bromstyrol should be excluded from preparations for infants (Richardson, 1937).

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Levenstein, I. (1972b). Report to RIFM, 7 April.
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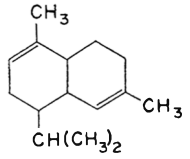
CADINENE

Synonym: 3,4,7,8,9,10-Hexahydro-4-isopropyl-1,6-dimethylnaphthalene.
Structure: α-Cadinene has the following structure but several isomers occur in nature with the double bond in various positions; unconjugated:



Description and physical properties: A colourless slightly viscous oil generally carrying the odour of the oil from which it is derived (Gildemeister & Hoffman, 1960).
Occurrence: Cadinene in its isomeric forms occurs in over 150 essential oils (Gildemeister & Hoffman, 1960).
Preparation: By isolation from various essential oils (Gildemeister & Hoffman, 1960).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 4000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0.001	0·01	0·04
Maximum	0·05	0·005	0·05	0·5

Analytical data: Gas chromatogram, RIFM no. 72–11; infra-red curve, RIFM no. 72–11

Status

Cadinene is approved by the FDA for food use (21 CFR 121.1164).

Biological data

Acute toxicity. The acute oral LD_{50} was reported to be > 5 g/kg in the rat (Keating, 1972). The acute dermal LD_{50} was reported to be > 5 g/kg in the rabbit (Keating, 1972).

Irritation. Cadinene tested at a concentration of 10% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

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

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Kligman, A. M. (1972). Report to RIFM, 19 July.

CAMPHOR OIL WHITE

Description and physical properties: A white viscous liquid with cineole as the principal component along with monoterpenes (Guenther, 1950).
Occurrence: Found in the trees and bark of *Cinnamomum camphora* Sieb (fam. Lauraceae).
Preparation: By fractional distillation of crude camphor oil after the camphor has been crystallized out (Arctander, 1960).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 15,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·01	0·04
Maximum	0·1	0·02	0·1	0·4

Status

Camphor oil white was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1163). The Council of Europe (1970) included camphor oil (*Cinnamomum camphora*) in the list of temporarily admissible flavouring substances (provided no safrole is present in the final product).

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported to be 5·10 ml/kg (2·73–7·47 ml/kg) (Hart, 1971). The acute dermal LD_{50} was reported to be > 5 ml/kg in the rabbit (Hart, 1971).

Irritation. Camphor oil white applied full strength to intact or abraded rabbit skin was mildly irritating (Hart, 1971). Camphor oil tested at a concentration of 20% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 20% in petrolatum and produced no sensitization reactions (Kligman, 1971).

Metabolism. Cineole, the principal ingredient of white camphor oil, undergoes oxidation *in vivo* with the formation of hydroxycineole, which is excreted as hydroxycineoleglucuronic acid (Williams, 1959).

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Kligman, A. M. (1971). Report to RIFM, 14 June.
Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed., p. 528. Chapman & Hall Ltd., London.

CANANGA OIL

Description and physical properties: EOA Spec. no. 75.
Occurrence: Found in the flowers of the tree *Cananga odorata* Hook. f. et Thomsen, syn *Canangium odoratum* Baill. forma macophylla (fam. Anonaceae).
Preparation: By the steam-distillation of the flowers of the cannaga tree.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·05	0·2
Maximum	0·2	0·02	0·2	0·8

Status

Cananga oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included cananga oil (*Cananga odorata*) in the list of temporarily admitted flavoring substances. The *Food Chemicals Codex* (1972) has a monograph on cananga oil.

Biological data

Acute toxicity. The acute oral LD_{50} was reported to be >5 g/kg in the rat (Hart, 1971). The acute dermal LD_{50} was reported to be >5 g/kg in the rabbit (Hart, 1971).

Irritation. Cananga oil applied full strength to intact or abraded rabbit skin was irritating (Hart, 1971). Cananga oil tested at a concentration of 10% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1971).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 2, no. 103, p. 16. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2232. *Fd Technol., Champaign* **19**(2), part 2, 155.
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Kligman, A. M. (1971). Report to RIFM, 14 June.

CARAWAY OIL

Description and physical properties: Food Chemicals Codex (1972).
The main constituent of caraway oil is *l*-carvone.
Occurrence: Found in the fruits of *Carum carvi* L. (fam. Umbelliferae)
Preparation: By steam-distillation of the dried ripe fruit of *Carum carvi* L.
Uses: In public use since the 1920s.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	—	0·01	0·02
Maximum	0·05	—	0·05	0·4

Analytical data: Gas chromatogram, RIFM no. 72–88; infra-red curve, RIFM no. 72–88.

Status

Caraway oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included caraway oil (*Carum carvi*) in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product. Both the *Food Chemicals Codex* (1972) and the National Formulary (1970) have monographs on caraway oil.

Biological data

Acute toxicity. The acute oral LD_{50} in the rats was reported to be 3·5 ml/kg (2·7–4·7 ml/kg) (Shelanski & Moldovan, 1972). The acute dermal LD_{50} in the rabbit was reported to be 1·78 ml/kg (1·46–2·18 ml/kg) (Shelanski & Moldovan, 1972).

Irritation. Undiluted caraway oil applied to the backs of hairless mice produced no irritating effects (Urbach, 1972). Caraway oil applied full strength to intact or abraded rabbit skin was irritating (Shelanski & Moldovan, 1972). Tested at a concentration of 4% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. Low-level phototoxic effects have been reported for caraway oil, but these are not considered significant (Urbach & Forbes, 1972).

References

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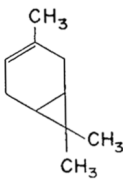
National Formulary (1970). 13th ed. Prepared by the National Formulary Board, p. 134. American Pharmaceutical Association. Washington, D.C.

Shelanski, M. V. & Moldovan, M. (1972). Report to RIFM, 14 July.

Urbach, F. & Forbes, P. D. (1972). Report to RIFM, 26 July.

Δ^3-CARENE

Synonyms: Isodiprene; 3,7,7-trimethylbicyclo-[*0,1,4*]-3-heptene.
Structure:



Description and physical properties: A colourless mobile liquid with a sweet odour (Arctander, 1969).
Occurrence: Δ^3-Carene has been identified in many volatile oils such as Swedish and Finnish turpentine oils, galanga root oil and in German pine needle oils such as those from *Pinus pumilio & Pinus sylvestris* (Guenther, 1949).
Preparation: By isolation from turpentine fractions (Arctander, 1969).
Uses: In public use since the 1940s.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·001	0·005
Maximum	0·1	0·05	0·005	0·01

Analytical data: Gas chromatogram, RIFM no. 72–12; infra-red curve, RIFM no. 72–12.

Specifications of sample no. 72–12: Specific gravity (25°/25°C), 0·8590; optical rotation (αD), +11·56; refractive index (n_D^{20}), 1·4733; soln in 90% alcohol, 5·5 vols, and in 95% alcohol, 2·0 vols; Δ^3-carene content (by GLC) 95·1%. This sample was collected and stored under nitrogen, washed several times and kept refrigerated under nitrogen.

Status

There is an extensive review of the chemistry of carene by Cocker (1971).

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported to be 4·8 g/kg (4·0–5·6 g/kg) (Moreno, 1972a). The acute dermal LD_{50} was reported to be > 5 g/kg in the rabbit (Moreno, 1972b).

Irritation. Δ^3-Carene applied full strength to intact or abraded rabbit skin produced irritation (Moreno, 1972b). Tested at a concentration of 10% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

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

Additional published data

The oxidation products of Δ^3-carene, which is an important constituent of oil of turpentine and is also present as an impurity in α-pinene, are responsible for the eczematogenic effect of oil of turpentine and of the terpenes distilled from it (Pirilä & Siltanen, 1956). Autoxidation of Δ^3-carene to the hydroperoxide forms the actual eczematogen in turpentine

oil (Pirilä & Siltanen, 1957). The eczematogenic effect of oil of turpentine is mainly due to the oxidation products (hydroperoxide) of Δ^3-carene (Pirilä, Siltanen & Pirilä, 1964).

Δ^3-Carene was shown to be a sensitizer in a study of the sensitizing potential of some essential oils and their constituents (Woeber & Krombach, 1969). Δ^3-Carene exerts an allergic effect upon the skin (Mikhailov & Berova, 1970). Applied undiluted to the skin of guinea-pigs, it produced skin reactions within 24 and 48 hr, consisting of widespread erythematous papular reactions with infiltration and desquamation (Hellerstrom, Lodin, Rajka, Swedin & Widmark, 1963). Of nine distillation fractions of turpentine oil, mainly Δ^3-carene and limonene were responsible for dermatitis (Lejhancová & Wolf, 1955).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, p. 570. S. Arctander, Montclair, New Jersey.

Cocker, W. (1971). A review of some investigations of the chemistry of carene. *J. Soc. cosmet. Chem.* **22**, 249.

Guenther, E. (1949). *The Essential Oils*. Vol. II. p. 49. D. Van Nostrand, Inc., Princeton, New Jersey.

Hellerstrom, S., Lodin, A., Rajka, G., Swedin, B. & Widmark, G. (1963). Sensitization of pigs with Δ^3-carene. *Acta derm.-vener., Stockh.* **43**(4), 311.

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Lejhancová, G. & Wolf, J. (1955). Effect of terpenes and their oxidation products on the skin. *Pracovní Lék.* **7**, 285.

Mikhailov, P. & Berova, N. (1970). Clinical and biochemical studies on turpentine contact allergy. *Dermato-Vener., Sofia* **19**(1), 20.

Moreno, O. M. (1972a). Report to RIFM, 1 May.

Moreno, O. M. (1972b). Report to RIFM, 5 May.

Pirilä, V. & Siltanen, E. (1956). On the chemical nature of the eczematous agent in oil of turpentine, II. *Dermatologica* **113**(1), 1.

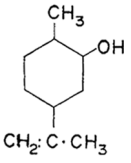
Pirilä, V. & Siltanen, E. (1957). On the Eczematous Agent in Oil of Turpentine. Proceedings of the International Congress on Occupational Health. Helsinki, on 6 & 7 January, **3**, 400.

Pirilä, V., Siltanen, E. & Pirilä, L. (1964). Chemical nature of the eczematogenic agent in oil of turpentine. IV. Primary irritant effect of terpenes. *Dermatologica* **128**(1), 16.

Woeber, K. & Krombach, M. (1969). Zur Frage der Sensibilisierung durch ätherische Öle. *Berufsdermatosen* **17**, 320.

l-CARVEOL

Synonyms: *l*-Methyl-4-isopropenyl-6-cyclohexen-2-ol; *l*-*p*-mentha-6,8-dien-2-ol.
Structure:



Description and physical properties: A colourless liquid with a spearmint-like odour (Arctander, 1969).
Occurrence: Found in small quantities in spearmint oil (Arctander, 1969).
Preparation: *l*-Carveol is produced from *l*-carvone by selective reduction (Arctander, 1969).
Uses: In public use since the 1940's. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

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

Analytical data: Gas chromatogram, RIFM no. 72–13; infra-red curve RIFM no. 72–13.

Status

Carveol was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included carveol in its list of temporarily admissible artificial flavouring substances.

Biological data

Acute toxicity. The acute oral LD_{50} in the rat was reported to be 3·0 g/kg (2·34–3·83 g/kg) (Keating, 1972). The acute dermal LD_{50} was reported to be >5 g/kg in the rabbit (Keating, 1972).

Irritation. *l*-Carveol applied full strength to intact or abraded rabbit skin produced irritation (Keating, 1972). Tested at a concentration of 4% in petrolatum, it produced no irritation in a 48-hr closed-patch test on 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1972).

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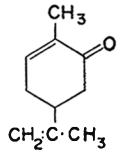
Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 2, no. 29, p. 95. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2247. *Fd Technol., Champaign* **19**(2), part 2, 155.
Keating, J. W. (1972). Report to RIFM, 7 June.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1972). Report to RIFM, 19 July.

l-CARVONE

Synonyms: *l*-6,8(9)-*p*-Menthadien-2-one; *l*-1-methyl-4-isopropenyl-6-cyclohexen-2-one.
Structure:



Description and physical properties: EOA Spec no. 131.
Occurrence: Found in a score of essential oils and is the main constituent of spearmint oil.
Preparation: By isolation from oil of spearmint or synthesized commercially from *d*-limonene.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	Trace	0·005	0·02
Maximum	0·05	Amounts	0·01	0·1

Analytical data: Gas chromatogram, RIFM no. 70–20; infra-red curve, RIFM no. 70–20.

Status

l-Carvone was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) listed carvone, giving an ADI of 1·25 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on *l*-carvone, and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for *l*-carvone, giving an ADI of 1·25 mg/kg.

Biological data

Acute toxicity. The acute oral LD_{50} was reported to be 1640 mg/kg in rats and 766 mg/kg in the guinea-pig (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964).

Chronic toxicity. In the feeding study in rats, 10,000 ppm fed in the diet for 16 wk caused growth retardation and testicular atrophy (Hagan, Hansen, Fitzhugh, Jenner, Jones Taylor, Long, Nelson & Brouwer, 1967), while 1000 ppm fed for 28 wk and 2500 ppm fed for 1 yr produced no effects.

Irritation. *l*-Carvone tested at a concentration of 1 % in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 1 % in petrolatum and produced no sensitization reactions (Kligman, 1971).

Metabolism. Carvone was metabolized by the rabbit to 1,5-dimethyl-1,5-hexadien-1,6-dicarboxylic acid and a carbinol in which one ethylene linkage was saturated and the keto group was reduced (Fischer & Bielig, 1940).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 146, p. 56. Strasbourg.

Fischer, F. G. & Bielig, H. J. (1940). Biochemical hydrogenations. VII. Hydrogenation of unsaturated compounds in the animal body. *Hoppe-Seyler's Z. physiol. Chem.* **266**, 73.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2249. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 178. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Hagan, E. C., Hansen, W. H., Fitzhugh, O. G., Jenner, P. M., Jones, W. I., Taylor, Jean M., Long, Eleanor L., Nelson, A. A. & Brouwer, J. B. (1967). Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Fd Cosmet. Toxicol.* **5**, 141.

Jenner, P. M., Hagan, E. C., Taylor, Jean M., Cook, E. L. & Fitzhugh, O. G. (1964). Food flavourings and compounds of related structure. I. Acute oral toxicity. *Fd Cosmet. Toxicol.* **2**, 327.

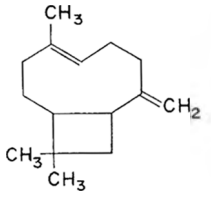
Joint FAO/WHO Expert Committee on Food Additives (1967). Toxicological Evaluation of Some Flavouring Substances and Non-nutritive Sweetening Agents. *F.A.O. Nutr. Mtg. Rep. Ser. no.* 44A, Geneva, p. 14. WHO/Food Add./68.33.

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

Kligman, A. M. (1971). Report to RIFM, 24 May.

CARYOPHYLLENE

Synonym: β-Caryophyllene
Structure:



Description and physical properties: Givaudan Index (1961).
Occurrence: Found in oils of clove, cinnamon leaves and copaiba balsam and in minor quantities in various other essential oils, especially lavender (*Givaudan Index*, 1961).
Preparation: By isolation from clove leaf oil, clove stem oil, cinnamon leaf oil or pine oil fractions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 20,000 lb/yr.

Concentration in final product (%):

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

Status

Caryophyllene was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included caryophyllene in the list of temporarily admissible artificial flavouring substances. The *Food Chemicals Codex* (1972) has a monograph on caryophyllene.

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 (Hart, 1971).

Irritation. Caryophyllene applied full strength to intact or abraded rabbit skin was irritating (Hart, 1971). Tested at a concentration of 4% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1971).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals* (*Aroma Chemicals*). Vol. 1, p. 584. S. Arctander, Montclair, New Jersey.

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Flavouring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2252. *Fd Technol., Champaign* **19** (2), part 2, 155.

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Hart, E. R. (1971). Report to RIFM, 30 July.

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

Kligman, A. M. (1971). Report to RIFM, 14 June.

CASTOREUM

Description and physical properties: EOA Spec no. 194. The material on which the testing was carried out was castoreum tincture.

Occurrence: Found as the secretion obtained from the oil glands of the beaver *Castor fiber* L. (Castoridae).

Preparation: By alcoholic extraction of the macerated castoreum pods of the beaver, a by-product of the fur industry (Arctander, 1960).

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

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	—	0·01	0·04
Maximum	0·05	—	0·1	0·4

Status

Castoreum was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included castoreum in the list of flavouring substances whose use is temporarily admitted, with a possible limitation on the active principle in the final product.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported to be > 5 g/kg (Keating, 1972). The acute dermal LD_{50} in the rabbit was reported to be > 5 g/kg (Keating, 1972).

Irritation. Undiluted castoreum tincture applied to the backs of hairless mice produced no irritation (Urbach & Forbes 1973). Castoreum tincture applied full strength to intact or abraded rabbit skin was not irritating (Keating, 1972). Castoreum tested at a concentration of 4% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. No phototoxic effects were reported for castoreum tincture (Urbach 1973).

References

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin.* 1st ed. p.136. S. Arctander, Elizabeth, New Jersey.

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 2, no. 2, p. 30. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2261. *Fd Technol., Champaign* **19**(2), part 2, 155.

Keating, J. W. (1972). Report to RIFM, 20 April.

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

Kligman, A. M. (1972). Report to RIFM, 12 June.

Urbach, F. & Forbes, P. D. (1973). Report to RIFM, 6 February.

CINNAMYL ACETATE

Synonym: 3-Phenyl-2-propen-1-yl acetate.
Structure: $C_6H_5 \cdot CH:CH \cdot CH_2 \cdot CO_2 \cdot CH_3$.
Description and physical properties: Food Chemicals Codex (1972).
Occurrence: Found in cassia oil and several other essential oils (*Givaudan Index*, 1961).
Preparation: By direct esterification of cinnamic alcohol with acetic acid (or anhydride) under azeotropic conditions (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·01	0·04
Maximum	0·1	0·01	0·1	0·5

Analytical data: Infra-red curve, RIFM no. 72–232.

Status

Cinnamyl 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 (1970) listed cinnamyl acetate, giving an ADI of 1·25 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on cinnamyl acetate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported to be 3·3 g/kg (2·9–3·7 g/kg) (Moreno, 1972). The acute dermal LD_{50} was reported to be > 5 g/kg in the rabbit (Moreno, 1972).

Irritation. Cinnamyl acetate applied full strength to intact or abraded rabbit skin produced no irritation (Moreno, 1972). Tested at a concentration of 5% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 5% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

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Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A (1), Series 1, no. 209, p. 60. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2293. *Fd Technol., Champaign* **19**(2), part 2, 155.
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Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1972). Report to RIFM, 13 October.
Moreno, O. M. (1972). Report to RIFM, 1 November.

CITRAL DIMETHYL ACETAL

Synonyms: *cis-* and *trans*-3,7-Dimethyl-1,1-dimethoxy-2,6-octadienes; *cis-* and *trans*-2,6-dimethyl-8,8-dimethoxy-2,6-octadiene.

Structure: $CH_3 \cdot C(CH_3):CH \cdot [CH_2]_2 \cdot C(CH_3):CH \cdot CH(OCH_3)_2$.

Description and physical properties: EOA Spec. no. 273.

Occurrence: Apparently has not been reported to occur in nature.

Preparation: From citral and methyl alcohol in the presence of a catalyst, or by reacting citral with trimethyl orthoformate.

Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 50,000 lb/yr.

Concentration in final product (%).

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·005	0·03	0·05
Maximum	0·15	0·02	0·15	0·4

Status

Citral dimethyl acetal was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed citral dimethyl acetal, giving an ADI of 5 mg/kg.

Biological data

Acute toxicity. Both the oral LD_{50} value in rats and the dermal LD_{50} value in rabbits exceeded 5 g/kg (Hart, 1971).

Irritation. Citral dimethyl acetal applied full strength to intact or abraded rabbit skin was irritating (Hart, 1971). Tested at a concentration of 4% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1971).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 40, p. 50. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2305. *Fd Technol., Champaign* **19**(2), part 2, 155.

Hart, E. R. (1971). Report to RIFM, 30 July.

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

Kligman, A. M. (1971). Report to RIFM, 14 June.

CITRONELLA OIL

Description and physical properties: EOA Spec. nos 12 and 14. The main constituents of citronella oil are geraniol and citronellal (Guenther, 1950).
Occurrence: Found in the grasses of *Cymbopogon Nardus* (Rendle), *Andropogon Nardus* (L) and *Andropogon Nardus* Ceylon, de Jong (Fam. Gramineae).
Preparation: By direct steam-distillation of the dried grass.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to about 400,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·01	0·03	0·2
Maximum	0·60	0·03	0·30	0·80

Analytical data: Gas chromatogram, RIFM no. 2008, 71–92, 71–93; infra-red curve, RIFM no. 2008, 71–92, 71–93.

Status

Citronella was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included citronella (*Cymbopogon Nardus*) in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation on the active principle in the final product.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as > 5 g/kg (Shelanski & Moldovan, 1971). The acute dermal LD_{50} in rabbits was reported as 4·7 ml/kg (3·4–6·7 ml/kg) (Shelanski & Moldovan, 1971).

Irritation. Citronella oil applied at full strength to intact or abraded rabbit skin caused irritation (Shelanski & Moldovan, 1971). Three different samples of citronella oil, RIFM nos 2008 (citronella Formosa), 71–92 (citronella Ceylon) and 71–93 (citronella Java), tested at a concentration of 8% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. Maximization tests (Kligman, 1966) carried out on 25 volunteers at a concentration of 8% in petrolatum using three different samples of citronella oil, RIFM nos 2008 (citronella Formosa), 71–92 (citronella Ceylon) and 71–93 (citronella Java) produced no sensitization reactions (Kligman, 1971).

Additional published data

Three cases of an eczematous, contact-type of hypersensitivity to oil of citronella have been reported (Keil, 1947). Folliculitis of the acneform type has been induced by citronella oil (Lane, 1922). A case of papulovesicular eczema of the hands, fingers and forearms, verified by a patch test with oil of citronella, has also been reported (Flandin, Rabeau & Ukrainczyk, 1937). Oil of citronella in perfumes is listed as a primary irritant and sensitizer by Schwartz & Peck (1946) and Schwartz, Tulipan & Peck (1947).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 39, p. 14. Strasbourg.

Flandin, C., Rabeau, H. & Ukrainczyk, A. (1937). L'intolerance a la terebenthine et aux substances du groupe des terpenes. *Bull. Soc. fr. Derm. Syph.* **44,** 315.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2308. *Fd Technol., Champaign* **19**(2), part 2, 155.
Guenther, E. (1950). *The Essential Oils.* Vol. IV, p. 256. D. Van Nostrand, Inc., Princeton, New Jersey.
Keil, H. (1947). Contact dermatitis due to oil of citronella. *J. invest. Derm.* **8,** 327.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47,** 393.
Kligman, A. M. (1971). Report to RIFM, 27 September and 3 November.
Lane, C. G. (1922). Dermatitis caused by oil of citronella. *Archs. Derm. Syph.* **5,** 589.
Schwartz, L. & Peck, S. M. (1946). *Cosmetics and Dermatitis.* Hoeber, New York.
Schwartz, L., Tulipan, L. & Peck, S. M. (1947). *Occupational Diseases of the Skin.* Lea and Febiger, Philadelphia.
Shelanski, M. V. & Moldovan, M. (1971). Report to RIFM, 14 November and 26 November.

CITRONELLYL ACETATE

Synonyms: 2,6-dimethyl-(1 or 2)-octene-8-yl acetate; 3,7-dimethyl-6-octen-1-yl acetate.
Structure: $CH_3 \cdot C(CH_3) : CH \cdot [CH_2]_2 \cdot CH(CH_3) \cdot [CH_2]_2 \cdot CO_2 \cdot CH_3$.
Description and physical properties: EOA Spec. no. 125.
Occurrence: Found in oils of citronella Ceylon, geranium and about twenty other oils (Gildemeister & Hoffman, 1966).
Preparation: By esterification of citronellol with acetic acid or acetic anhydride.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·03	0·12
Maximum	0·10	0·01	0·10	0·40

Analytical data: Gas chromatogram, RIFM no. 70–40; infra-red curve, RIFM no. 70–40.

Status

Citronellyl 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 (1970) listed citronellyl acetate, giving an ADI of 0·25 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on citronellyl acetate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported to be 6·8 g/kg (Calandra, 1971). The acute dermal LD_{50} in rabbits was reported to be >2 g/kg (Calandra, 1971).

Irritation. Citronellyl acetate applied full strength to intact or abraded rabbit skin was irritating (Calandra, 1971). Tested at a concentration of 4% in petrolatum, it produced a mild irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1971).

References

Calandra, J. C. (1971). Report to RIFM, 12 April.
Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 203, p. 59. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2311. *Fd Technol., Champaign* **19**(2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 208. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.
Gildemeister, E. & Hoffman, F. (1966). *Die Ätherischen Öle.* Vol. IIId. p. 245. Akademie Verlag, Berlin.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1971). Report to RIFM, 25 March.

CITRONELLYL *n*-BUTYRATE

Synonyms: 3,7-Dimethyl-6-octen-1-yl *n*-butyrate; 2,6-dimethyl-2-octen-8-yl *n*-butyrate.
Structure: $CH_3 \cdot C(CH_3) : CH \cdot [CH_2]_2 \cdot CH(CH_3) \cdot [CH_2]_2 \cdot CO_2 \cdot [CH_2]_2 \cdot CH_3$.
Description and physical properties: Food Chemicals Codex (1972).
Occurrence: Found in oil of citronella Ceylon and the leaf oil of *Phebalium dentatum* (Gildemeister & Hoffman, 1966).
Preparation: By direct esterification of citronellol with butyric acid under azeotropic conditions or by treatment with butyric anhydride (Gildemeister & Hoffman, 1966).
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	0·04
Maximum	0·05	0·005	0·05	0·4

Analytical data: Infra-red curve, RIFM no. 72–104.

Status

Citronellyl butyrate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed citronellyl butyrate, giving an ADI of 0·25 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on citronellyl butyrate.

Biological data

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

Irritation. Citronellyl butyrate applied full strength to intact or abraded rabbit skin was not irritating (Moreno, 1972). Tested at a concentration of 5% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 5% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 276, p. 64. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2312. *Fd Technol., Champaign* **19**(2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 209. National Academy of Sciences-National Research Council Publ. 1406, Washington, D.C.
Gildemeister, E. & Hoffman, F. (1966). *Die Ätherischen Öle.* Vol. IIId. p. 270. Akademie Verlag, Berlin.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1972). Report to RIFM, 13 October.
Moreno, O. M. (1972). Report to RIFM, 1 November.

CITRONELLYL FORMATE

Synonyms: 2,6-Dimethyl-(1-or-2)-octen-8-yl formate; 3,7-dimethyl-6-octen-yl formate.
Structure: $CH_3 \cdot C(CH_3) : CH \cdot [CH_2]_2 \cdot CH(CH_3) \cdot [CH_2]_2 \cdot CO_2 \cdot H$.
Description and physical properties: EOA Spec. no. 206.
Occurrence: Found in nature in geranium oil.
Preparation: By esterification of citronellol with formic acid.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 40,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·04
Maximum	0·05	0·05	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 70–41; infra-red curve, RIFM no. 70–41.

Status

Citronellyl formate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed citronellyl formate giving an ADI of 0·25 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on citronellyl formate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported to be 8·4 g/kg (Calandra, 1971). The acute dermal LD_{50} in rabbits was reported to be >2 g/kg (Calandra, 1971).

Irritation. Citronellyl formate applied full strength to intact or abraded rabbit skin was irritating (Calandra, 1971). Tested at a concentration of 4% in petrolatum, it produced a mild irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1971).

References

Calandra, J. C. (1971). Report to RIFM, 12 April.
Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 346, p. 68. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2314. *Fd Technol., Champaign* **19**(2), part 2, 155.
Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 210. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1971). Report to RIFM, 25 March.

COPAIBA OIL

Description and physical properties: EOA Spec. no. 10. The principal constituent of copaiba oil is caryophyllene (Guenther, 1952). It also contains other sesquiterpenes (Gildemeister & Hoffman, 1959).

Occurrence: Found in the exudation from the trunk of the Copaifera L. (fam. Leguminosae).

Preparation: By the steam-distillation of copaiba balsam.

Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 33,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·05	0·20
Maximum	0·20	0·02	0·20	0·80

Status

Copaiba oil is approved by the FDA for food use (21 CFR 121.1163). The *Food Chemicals Codex* (1972) has a monograph on copaiba oil.

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 (Hart, 1971).

Irritation. Copaiba oil applied full strength to intact or abraded rabbit skin was irritating (Hart, 1971). Tested at a concentration of 8% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

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 (Kligman, 1971).

References

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 218. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Gildemeister, E. & Hoffman, F. (1959). *Die Ätherischen Öle.* Vol. V. p. 311. Akademie Verlag, Berlin.

Guenther, E. (1952). *The Essential Oils.* Vol. V. p. 204. D. Van Nostrand, Inc., Princeton, New Jersey.

Hart, E. P. (1971). Report to RIFM, 18 June.

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

Kligman, A. M. (1971). Report to RIFM, 9 June.

CORIANDER OIL

Description and physical properties: Food Chemicals Codex (1972). The main constituent of coriander oil is *d*-linaloöl (Guenther, 1950).

Occurrence: Found in the fruit of *Coriandrum sativum* L. (Fam. Umbelliferae).

Preparation: By steam-distillation of the dried ripe fruit.

Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	—	0·02	0·04
Maximum	0·05	—	0·06	0·6

Status

Coriander oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1970) included coriander oil (*Coriandrum sativum*) in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product. The *Food Chemicals Codex* (1972) and the United States Pharmacopeia (1965) have monographs on coriander oil.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported to be 4·13 g/kg (2·48–6·14 g/kg) (Hart, 1971). The acute dermal LD_{50} in rabbits was reported to be >5 g/kg (Hart, 1971).

Irritation. Coriander oil applied full strength to intact or abraded rabbit skin was irritating (Hart, 1971). Tested at a concentration of 6% in petrolatum, it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

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

References

Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 154, p. 18. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2334. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 220. National Academy of Sciences-National Research Council Publ. 1406, Washington, D.C.

Guenther, E. (1950). *The Essential Oils.* Vol. IV, p. 602. D. Van Nostrand, Inc., Princeton, New Jersey.

Hart, E. P. (1971). Report to RIFM, 30 July.

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

Kligman, A. M. (1971). Report to RIFM, 9 June.

United States Pharmacopeia (1965). 17th revision. Prepared by the Committee of Revision. p. 146. The United States Pharmacopeial Convention, Inc., New York.

SUPPLEMENT TO EARLIER MONOGRAPHS ON FRAGRANCE RAW MATERIALS*

Acetate C-9

Irritation. Acetate C-9 applied full strength to intact or abraded rabbit skin was not irritating (Levenstein, 1972). Tested in a concentration of 2% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Kligman, A. M. (1972). Report to RIFM, 27 March.
Levenstein, I. (1972). Report to RIFM, 7 April.

Acetophenone

Irritation. Undiluted acetophenone tested on rabbit skin is capable of causing irritation (Rowe & Wolf, 1963). Acetophenone tested at a concentration of 2% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Kligman, A. M. (1971). Report to RIFM, 9 June.
Rowe, V. K. & Wolf, M. A. (1963). Ketones. In *Industrial Hygiene and Toxicology*. 2nd ed. Edited by F. A. Patty. Vol. II, p. 1763. Interscience Publishers, New York.

Alcohol C-8

Irritation. Alcohol C-8 applied full strength to intact or abraded rabbit skin produced a mild irritation (Levenstein, 1972). Tested in a concentration of 2% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Kligman, A. M. (1972). Report to RIFM, 2 May.
Levenstein, I. (1972). Report to RIFM, 13 January.

Alcohol C-9

Irritation. Alcohol C-9 undiluted produced no irritation in the rabbit (Treon, 1963). Tested in a concentration of 2% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Kligman, A. M. (1972). Report to RIFM, 2 May.
Treon, J. F. (1963). Alcohols. In *Industrial Hygiene and Toxicology*. 2nd ed. Edited by F. A. Patty. Vol. II. p. 1464. Interscience Publishers, New York.

Alcohol C-10

Irritation. Alcohol C-10 undiluted produced a severe irritation in the rabbit after 24 hr (Treon, 1963). Tested in a concentration of 3% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Kligman, A. M. (1972). Report to RIFM, 27 March.
Treon, J. F. (1963). Alcohols. In *Industrial Hygiene and Toxicology*. 2nd ed. Edited by F. A. Patty. Vol. II. p. 1467. Interscience Publishers, New York.

Alcohol C-11

Irritation. Alcohol C-11 applied full strength to intact or abraded rabbit skin produced a mild irritation (Levenstein, 1972). Tested in a concentration of 1% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Kligman, A. M. (1972). Report to RIFM, 14 March.
Levenstein, I. (1972). Report to RIFM, 13 January.

*Addenda to monographs published in *Food and Cosmetics Toxicology* (1973, **11**, 95 & 477).

Alcohol C-12

Irritation. Alcohol C-12 undiluted was practically non-irritating to the guinea-pig (Treon, 1963). Tested in a concentration of 4% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Kligman, A. M. (1972). Report to RIFM, 2 May.
Treon, J. F. (1963). Alcohols. In *Industrial Hygiene and Toxicology*. 2nd ed. Edited by F. A. Patty. Vol. II. p. 1468. Interscience Publishers, New York.

Aldehyde C-6

Irritation. Aldehyde C-6 undiluted was slightly irritating to rabbit skin (Fassett, 1963). Tested in a concentration of 1% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Fassett, D. W. (1963). Aldehydes and acetals. In *Industrial Hygiene and Toxicology*. 2nd ed. Edited by F. A. Patty, Vol. II. p. 1967. Interscience Publishers, New York.
Kligman, A. M. (1972). Report to RIFM, 27 March.

Aldehyde C-8

Irritation. Aldehyde C-8 undiluted produced a mild irritation in the rabbit (Smyth, Carpenter, Weil, Pozzani & Streigel, 1962).

Smyth, H. F., Jr., Carpenter, C. P., Weil, C. S., Pozzani, U. C. & Streigel, Jean A. (1962). Range-finding toxicity data: List VI. *Am. ind. Hyg. Ass. J.* **23**, 95.

Aldehyde C-9

Irritation. Aldehyde C-9 applied full strength to intact or abraded rabbit skin was severely irritating (Shelanski, 1971). Tested in a concentration of 1% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Kligman, A. M. (1971). Report to RIFM, 27 September.
Shelanski, M. V. (1971). Report to RIFM, 14 November.

Aldehyde C-10

Irritation. Aldehyde C-10 undiluted produced a mild irritation in the rabbit (Smyth, Carpenter, Weil, Pozzani & Striegel, 1962).

Smyth, H. F., Jr., Carpenter, C. P., Weil, C. S., Pozzani, U. C. & Striegel, Jean A. (1962). Range-finding toxicity data: List VI. *Am. ind. Hyg. Ass. J.* **23**, 95.

Aldehyde C-11, undecylenic

Irritation. Aldehyde C-11, undecylenic, applied full strength to intact or abraded rabbit skin was mildly irritating (Hart, 1971). Tested in a concentration of 1% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Hart, E. T. (1971). Report to RIFM, 18 June.
Kligman, A. M. (1971). Report to RIFM, 3 November.

Aldehyde C-11, undecylic

Irritation. Aldehyde C-11, undecylic, applied full strength to intact or abraded rabbit skin was mildly irritating (Shelanski, 1971). Tested in a concentration of 5% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Kligman, A. M. (1971). Report to RIFM, 27 September.
Shelanski, M. V. (1971). Report to RIFM, 14 November.

Aldehyde C-12, Lauric

Irritation. Aldehyde C-12, lauric, applied full strength to intact or abraded rabbit skin was moderately irritating (Calandra, 1971). Tested in a concentration of 1% in petrolatum it produced a mild irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1970).

Calandra, J. C. (1971). Report to RIFM, 12 April.
Kligman, A. M. (1970). Report to RIFM, 2 December.

Aldehyde C-12, MNA

Irritation. Aldehyde C-12, MNA, tested in a concentration of 4% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Kligman, A. M. (1971). Report to RIFM, 17 June.

Aldehyde, C-14, myristic

Irritation. Aldehyde C-14, myristic, applied full strength to intact or abraded rabbit skin was moderately irritating (Lynch, 1971). Tested in a concentration of 1% in petrolatum it produced no irritation in a 48 hr closed-patch test in 25 human subjects (Kligman, 1971).

Kligman, A. M. (1971). Report to RIFM, 17 June.
Lynch, T. A. (1971). Report to RIFM, 16 June.

Allyl caproate

Irritation. Allyl caproate applied full strength to intact or abraded rabbit skin was not irritating (Shelanski, 1971). Tested in a concentration of 4% in petrolatum it produced a mild irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Kligman, A. M. (1971). Report to RIFM, 21 September.
Shelanski, M. V. (1971). Report to RIFM, 26 November.

Allyl cyclohexyl propionate

Irritation. Allyl cyclohexyl propionate tested in a concentration of 4% in petrolatum produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Kligman, A. M. (1971). Report to RIFM, 3 November.

Allyl α-ionone

Irritation. Allyl α-ionone applied full strength to intact or abraded rabbit skin was mildly irritating (Shelanski, 1971). Tested in a concentration of 10% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Kligman, A. M. (1972). Report to RIFM, 21 February.
Shelanski, M. V. (1971). Report to RIFM, 14 November.

Amyl benzoate

Irritation. Amyl benzoate applied full strength to intact or abraded rabbit skin was mildly irritating (Weir, 1971). Tested in a concentration of 6% in petrolatum it produced no irritation in a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Kligman, A. M. (1972). Report to RIFM, 3 November.
Weir, R. J. (1971). Report to RIFM, 25 August.

Fd Cosmet. Toxicol. Vol. 11, pp. 1083–1095. Pergamon Press 1973. Printed in Great Britain

Review Section

Selenium in Relation to Dental Caries

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Introduction

It is well known that the level of susceptibility to dental caries in man is influenced by a number of intrinsic and extrinsic factors. An important element in this respect is the degree of resistance of the tooth against the attacking force. The resistance of the tooth, in turn, depends to a considerable extent upon its chemical composition, which is determined mainly during the period of tooth development. The role played by trace elements in determining the degree of resistance to dental caries has not been investigated adequately (Hadjimarkos, 1968). Although the enamel of teeth, where caries begins, contains at least 25 trace elements, only the influence of fluoride has been studied thoroughly. Trace elements may alter caries susceptibility by changing the chemical composition either of the inorganic or of the organic components of dental enamel during the time teeth are formed. On the other hand, trace elements may affect caries after the eruption of the teeth by influences exerted through the oral environment.

In the last two decades or so, a substantial amount of evidence has accumulated from a number of studies demonstrating that the trace element selenium increases dental caries in man and experimental animals when it is consumed during the period of development of the teeth and incorporated into their structure. The purpose of this paper is to review the various studies so far reported in the literature on the selenium–dental caries relationship and thus to present a comprehensive and unified picture of our knowledge on this subject.

Epidemiological studies on selenium and caries in children

The possibility that an increased intake of dietary selenium may be implicated in dental caries was initially suggested from the results of two studies, conducted over 35 years ago, among individuals living in seleniferous rural areas of the States of Nebraska, Wyoming and South Dakota (Smith, Franke & Westfall, 1936; Smith & Westfall, 1937). Soils and plants in extensive areas of these States were reported to contain high levels of selenium, and selenium poisoning in farm animals has been widespread. One of the most common symptoms of disease observed among the individuals examined was a high prevalence of dental caries.

In recent years, four independent epidemiological studies have been conducted on the effects of dietary selenium on the prevalence of caries in children. Three of these studies

included children living in the States of Oregon, Wyoming, South Dakota and Montana. The fourth study was carried out in New Zealand. Since children in all four studies were native born and reared in their respective areas, the effect of the increased consumption of selenium on caries is related to the developmental period of the teeth. No human studies have been undertaken on the possible influence on caries susceptibility of selenium consumed after the eruption of the teeth.

The Oregon study

The first comprehensive epidemiological studies on dietary selenium and caries were carried out among Oregon school children, 14–16 years old, living east (Klamath county) and west (Jackson and Josephine counties) of the Cascade Mountains (Hadjimarkos & Bonhorst, 1961; Hadjimarkos, Storvick & Remmert, 1952). The results are presented in Table 1 and demonstrate that a high prevalence of caries is directly related to high levels of selenium in 24-hour urine specimens, which reflect the intake from the daily diet. Further, the mean concentration of selenium in samples of eggs and milk produced and consumed in farms located in the high-caries areas of Oregon was about ten times greater than that in the same products collected from the low-caries region of the State. The levels of selenium in drinking-waters in both high- and low-selenium areas were insignificant. Contrary to popular belief, it is well known that in comparison with foods, drinking-water in general is not an important source of trace element ingestion in man, except for fluoride (Hadjimarkos, 1966a & 1967a). The fluoride content of drinking-waters used by the children in the Oregon study, as well as their dietary habits and the degree of hardness of water supplies, were investigated but were not responsible for the observed differences in the prevalence of caries.

Table 1. *Prevalence of dental caries among school children in Oregon and levels of selenium in urine, eggs, milk and water**

Variables	Low-caries area	High-caries area	
	Klamath Co.	Jackson Co.	Josephine Co.
DMF teeth/child	9·0	13·4	14·4
Se levels (ppm) in:			
Urine	0·037	0·074	0·076
Eggs	0·056	0·502	0·437
Milk	0·005	0·049	0·067
Water	Trace	0·001	0·002

DMF = Mean no. of decayed, missing and filled teeth

*Data from Hadjimarkos *et al.* (1952) and Hadjimarkos & Bonhorst (1961).

The Wyoming study

The prevalence of caries was investigated among children, 10–18 years old, born and reared in high- and low-selenium localities of Wyoming (Tank & Storvick, 1960). This State is known for its extensive areas of soils and vegetation containing high amounts of selenium. Since numerous water supplies in Wyoming contain high levels of fluoride, the results of the study were reported in respect of both selenium and fluoride intake. The data in Table 2 demonstrate that children in high-selenium localities of the State had an increased preva-

lence of caries compared with controls, regardless of whether they used drinking-water high or low in fluoride. From the results in Table 2 it seems that ingestion of increased levels of dietary selenium by children may reduce the well-known beneficial effects of fluoride on caries. The prevalence of caries among children in areas that were high in both selenium and fluoride was 41 % greater compared with that of subjects living in localities high in fluoride but low in selenium.

Table 2. *Prevalence of dental caries among school children in Wyoming living in high- and low-selenium regions**

Areas	DMF/child	Difference in caries (%)†
Low selenium + low fluoride	8.5	
High selenium + low fluoride‡	10·5	18
Low selenium + high fluoride	4·6	
High selenium + high fluoride§	7·8	41

DMF = Mean no. of decayed, missing and filled teeth

*Data from Tank & Storvick (1960).
†Compared with areas with comparable fluoride but low selenium levels.
‡Low fluoride in drinking-waters: 0–0·5 ppm.
§High fluoride in drinking-waters: 0·6–2·6 ppm.

The Montana–Oregon–South Dakota study

Dental examinations were carried out among children, 12–14 years of age, living in small towns located in high- and low-selenium areas in the States of Montana, Oregon and South Dakota (Ludwig & Bibby, 1969). The towns in the high-selenium areas were situated on soils where the median selenium content of the local forage plants was 0·26 ppm, whereas the towns in the low-selenium areas were situated on soils on which forage plants had a median concentration of only 0·025 ppm.

The investigators reported that the prevalence of caries among the children born and living continuously in the towns of the high-selenium areas was significantly higher than that among controls living in the low-selenium towns. Further, it was found that the mean selenium content of samples of cows' milk produced and consumed in the high-selenium areas was three times greater than that of samples collected from the low-selenium areas, 0·045 and 0·016 ppm, respectively.

The New Zealand study

The effect of dietary selenium intake on caries was studied in two groups of children, 5–14 years of age, living in rural areas of the South Island of New Zealand (Cadell & Cousins, 1960). It was reported that there was no difference in the prevalence of caries among the two groups. However, the difference between the mean concentrations of urinary selenium in the two groups of children, 0·021 and 0·030 ppm, was statistically significant. On the basis of this, the investigators concluded that there was correlation between caries and selenium intake.

However, it should be emphasized that the study was conducted in a region of New Zealand where widespread areas of selenium deficiency are known to exist (Hartley &

Grant, 1961; Rosenfeld & Beath, 1964). This is borne out by the fact that the mean levels of selenium in the urine samples collected from the two groups of children were 0·021 and 0·030 ppm. Both figures are below the normal level of urinary selenium, which is considered to be 0·035–0·040 ppm (Glover, 1967 & 1970), and also below the urinary selenium levels found in the low-caries and low-selenium area of the study in Oregon children discussed above (Hadjimarkos *et al.* 1952; Hadjimarkos & Bonhorst, 1961). As pointed out elsewhere (Hadjimarkos, 1960a), the New Zealand investigators assumed erroneously that selenium increases caries at all levels of intake.

Experimental animal studies

In order to investigate the effect of selenium on caries when it is ingested either during the period of tooth development (systemic effect) or after the eruption of the teeth (local effect), a number of studies with laboratory animals have been conducted utilizing both experimental approaches.

Developmental period

Buttner (1963) administered selenium as sodium selenite to two groups of pregnant rats and to their pups in drinking-water at levels of 2·3 and 4·6 ppm. The results are presented in Table 3 and demonstrate that the pups that were exposed to dietary selenium during the entire period of the development of their teeth, *in utero* and postnatally, developed significantly more caries than did the controls. Rats on 2·3 and 4·6 ppm levels of selenium developed, respectively, 36 and 62% more carious lesions than the controls. The extent of carious lesions in groups given selenium at levels of 2·3 and 4·6 ppm was increased by 61 and 117%, respectively, in comparison with the controls. Administration of selenium at both levels reduced the number of young born and also decreased weight gains as a result of reduced food intake. However, despite the fact that rats on selenium consumed much less caries-producing food they developed more caries than the controls consuming the normal amount.

Table 3. *Effect of selenium on dental caries in the rat**

Selenium added to water (ppm)	No. of rats		Body-weight gain		Carious lesions	
	M	F	M	F	Mean no./rat†	Extent of lesions†
0	17	14	330 ± 9	207 ± 7	5·3 ± 0·5	11·6 ± 1·4
2·3	13	7	240 ± 5	176 ± 7	7·2 ± 0·7 (2·5)	18·7 ± 2·2 (3·0)
4·6	5	2	220 ± 18	148 ± 3	8·6 ± 1·1 (2·8)	25·2 ± 3·2 (4·0)

*Data from Buttner (1963).
†Sexes combined.
Values are given as means ± SD, with *t* values in parentheses, where appropriate.

In a recent study (Bowen, 1972), three young monkeys (*Macaca irus*) were given drinking-water containing selenium as sodium selenate for a period of approximately 5 years. The amount of selenium initially added to the water was at the level of 2 ppm. However, because of mild symptoms of toxicity the level of selenium was reduced a few months later to 1

ppm. The results of this study are presented in Table 4 and demonstrate that monkeys on selenium developed twice as many carious lesions as the controls, the increase being confined to the teeth that were formed during the ingestion of selenium in water. The data in Table 4 also show that the development of carious lesions in the teeth formed at the time selenium was consumed was much faster than that in the control monkeys, the mean time required for caries to develop in the selenium-treated monkeys being 6·7 months compared with 21·0 months in the controls.

Table 4. *Effect of selenium on dental caries in monkeys**

Variable	Selenium-treated monkeys	Controls
No. of animals	3	7
No. of carious lesions	48	59
Mean no. of lesions	16·0	8·4
Mean time (months) for caries development	6·7†	21·0

*Data from Bowen (1972).
†Time required for caries to develop in teeth exposed to selenium during the period of their development.

Post-eruptive period

The influence of selenium on caries after the eruption of the teeth has been studied in experimental animals with contradictory results. Selenium given post-eruptively was either ineffective or resulted in an increase or decrease in caries (Hadjimarkos, 1968). These inconsistent results were apparently due to the well-known fact that the development of caries in experimental animals is influenced by a variety of factors (Hadjimarkos, 1968; Buttner, 1969).

In recent years, it has also been demonstrated (Hadjimarkos, 1966b & 1967b) that addition of increased levels of selenium to the drinking-water of experimental animals alters the physiological ratio of food-to-water intake and also decreases food and water consumption, all of which have a decisive influence on the development of carious lesions. Even in cases where food consumption and weight gains by animals on selenium remain similar to those of the controls, there is still a significant reduction in water intake because of the objectionable taste and/or odour of selenium added to the water (Hadjimarkos, 1970a,b). In view of this, the decrease in caries observed in a study of rats given drinking-water containing 4·5 ppm selenium was attributed to a substantial reduction in the amount of caries-producing diet consumed by the animals (Konig, Savdir, Marthaler, Schmid & Muhlemann, 1964). In the study with monkeys referred to above (Bowen, 1972), a mild decrease in caries was seen in the first molars, which were formed before the animals were given drinking-water containing selenium. It is believed that this mild reduction in caries was produced by a decreased intake of drinking-water, resulting from the offensive taste and/or odour of selenium present in it, and this from a decreased intake of sugar which was added to the drinking-water at a level of 3% to accelerate the development of caries. Thus from all the above data it appears that the use of experimental animals for studying the post-eruptive effect of selenium on dental caries is not feasible.

Mechanism of action of selenium in caries

Selenium is known to be readily incorporated into the protein fraction of many tissues, and it is generally believed that it replaces sulphur in the sulphur-containing amino acids, occurring as selenocystine and selenomethionine (Underwood, 1971). Although selenium has been established as a normal constituent of human teeth (Hadjimarkos & Bonhorst, 1959 & 1960; Nixon & Myers, 1970), its site in dental tissues has not been investigated experimentally.

In a recent study, four pregnant rats each received a total dose of 48 μCi $Na_2{}^{75}SeO_3$, which was divided into four injections given between days 10 and 19 of pregnancy (Shearer & Hadjimarkos, 1973). On post-partum day 13, the mothers and their pups were sacrificed and radioactivity was measured in the developing teeth of the pups and in the fully formed and erupted teeth of their mothers. The results presented in Table 5 demonstrate that the molars, incisors and mandibles of the pups retained considerably more selenium than the same organs in their mothers. It is noteworthy that the developing and unerupted first and second molars of the pups retained about 43 times more ^{75}Se than the three fully formed molars of the mothers.

Table 5. *Distribution of retained ^{75}Se in the teeth and mandible of mother rats and their pups**

		Retained ^{75}Se/organ (%)†	
Tissue	No. of animals . . .	Pups 39	Mothers 4
Molars		0·13 ± 0·002	0·003 ± 0·001
Incisors		0·10 ± 0·002	0·041 ± 0·002
Mandible		0·32 ± 0·012	0·107 ± 0·007

*Data from Shearer & Hadjimarkos (1973).
†Calculated from the total ^{75}Se present in carcass (including head) and soft tissues = 100%.
Values are expressed as means ± SEM for the no. of rats indicated.

The location of the developmental and post-eruptive uptake of ^{75}Se in the crowns of the molar teeth of pups and their mothers was studied by separating the enamel and dentine fractions and measuring the ^{75}Se content of these two tissues. The data presented in Table 6 show that the enamel and dentine of the developing and unerupted molars of the pups incorporated considerably more selenium than did these two tissues from the mature and erupted molars of the mothers. In a further step, the protein component was isolated from the inorganic portion of enamel and dentine and radioactivity was measured in the protein fraction of the two tissues. The results revealed that 70% of the ^{75}Se present in the enamel and 94% of that in the dentine was in the protein fraction.

The demonstration that most of the selenium is incorporated into the protein components of enamel and dentine during the development of the teeth favours the belief that selenium increases caries by interfering with crystal nucleation and growth, thus inhibiting mineralization, as has been suggested elsewhere (Eisenmann & Yaeger, 1969; Newesely, 1970). In support of this mechanism, it is of considerable interest to note that a single injection of a

Table 6. *Distribution of ^{75}Se in the molar teeth of rats**

Dental tissue	^{75}Se level (% of dose†/100 g tissue)	
	Pups (developmental uptake)	Mothers (post-developmental uptake)
Whole tooth powder	7·13 ± 0·11 (9)	0·75 ± 0·21 (2)
Enamel	1·36 ± 0·05 (9)	0·15 ± 0·01 (2)
Dentine	7·40 ± 0·10 (8)	0·97 ± 0·31 (2)

*Data from Shearer & Hadjimarkos (1973).
†Each pregnant rat was injected with a total dose of 48 μCi $Na_2{}^{75}SeO_3$.
Values are expressed as means ± SEM, the no. of pools being indicated in parentheses. For the pups, each pool was made from all the unerupted first and second molar teeth from four litter-mate pups, or a total of 32 teeth for each pool. For the mothers, each pool was made from all the erupted first, second and third molar teeth from two rats, or a total of 24 teeth for each pool.

small amount of selenium into rats inhibited mineralization in the enamel and dentine of the continuously growing incisor teeth (Eisenmann & Yaeger, 1969). Moreover, it has been reported that administration of selenium to dogs and monkeys at the time their teeth were formed produced structural alterations in the enamel (Bowen, 1972; English, 1949).

Selenium–fluoride interaction

As was mentioned earlier, the epidemiological study among children in the State of Wyoming suggested that increased consumption of dietary selenium may reduce the beneficial effects of fluoride on caries (Tank & Storvick, 1960). However, the findings of a later study using rats indicated that the possible harmful effects of selenium on the caries-preventing action of fluoride could not be the result of a decreased uptake of fluoride by teeth (Hadjimarkos, 1967c). The amount of fluoride retained in the femurs of rats receiving 50 ppm fluoride and 3 ppm selenite-selenium in their drinking-water was similar to that of the controls given only 3 ppm selenite-selenium in the water.

Moxon & DuBois (1939) showed that the simultaneous administration of selenium (as seleniferous wheat) and fluoride to rats increased the manifestations of selenium toxicity. In a recent experiment, however, there was no increase in the appearance of symptoms of selenium intoxication in rats given 50 ppm fluoride and 3 ppm selenite-selenium in their drinking-water compared with control animals receiving only 3 ppm selenite-selenium (Hadjimarkos, 1969). It thus seems that among people living in seleniferous areas ingestion of the optimum amount of fluoride for caries prevention is not likely to enhance the possible ill-effects resulting from increased consumption of dietary selenium.

While the limited evidence from laboratory animals discussed above indicates that selenium intake does not diminish the beneficial effect of fluoride on caries or that fluoride intake does not enhance the possible detrimental effects of selenium on human health, it is felt that additional investigations are needed, particularly epidemiological studies among children, for a better understanding of the selenium–fluoride interaction.

Metabolism of selenium in relation to caries

Selenium is widespread in nature and small amounts of it are present in a variety of foodstuffs included in the daily diet. While the metabolism of selenium, as well as selenium

poisoning, has been studied extensively in farm and laboratory animals, "the research required on human phases of the problem has barely been touched", according to Rosenfeld & Beath (1964). With the exception of the harmful effects of selenium on dental caries, the possible dangers to the health of people living in the seleniferous areas known to exist in the USA and other countries and consuming locally produced foods have been largely ignored. In this connexion, it should be emphasized that a variety of common vegetables and plants cultivated on soils containing moderate amounts of selenium absorbed and stored amounts of this trace element that were potentially harmful to the health of people eating these products (Hamilton & Beath, 1963 & 1964).

Selenium in urine

Determination of selenium in urine samples provides a reliable index of man's intake from the total daily diet. It appears that the margin of safety between physiological levels and minimal toxic levels of selenium intake is narrow. The normal concentration of selenium in human urine is believed to be 0·035–0·040 ppm; levels above 0·1 ppm are considered dangerous to human health and are indicative of chronic selenium toxicity (Glover, 1967 & 1970; Lemley & Merryman, 1941; Sterner & Lidfeldt, 1941). However, it should be realized that levels of urinary selenium between the normal (0·035–0·040 ppm) and that which is considered toxic ($>0·1$ ppm) may not necessarily be harmless to man. Although such low levels of selenium ingestion may not be capable of producing obvious clinical manifestations of ill health, they may cause symptoms of disease which are subclinical in nature, and difficult to diagnose, as is known to be the case with other disease conditions (*Lancet*, 1973). The increase in dental caries susceptibility is believed to fall within this category of dose–response relationship, as is evident from the already discussed data on urinary selenium levels and the prevalence of caries among children in Oregon (Table 1). It should be noted also that according to the recommendations of the US Public Health Service (Code of Federal Regulations, Title 42, 1967), levels of selenium in drinking-waters exceeding 0·01 ppm are potentially dangerous to health and should not be used for human consumption.

Selenium in nails

In conducting epidemiological studies among children on selenium intake and the prevalence of caries, obvious difficulties arise in the collection, preservation and shipment of large numbers of urine samples. The results of a recent study indicate for the first time that the selenium content of human nail clippings, instead of urine, may serve as a reliable and convenient indicator of the intake of dietary selenium by man (Hadjimarkos & Shearer, 1973a).

The findings of the selenium concentration in samples of nail clippings and also of urine collected from 16 individuals aged between 21 and 53 years are presented in Table 7. The mean selenium content of nail clippings was 1·14 ppm, which is one of the highest levels ever recorded for human tissues. On the other hand, the mean urinary selenium level of 0·049 ppm among the participants appeared to be within normal limits, so that the mean selenium level in nail clippings should also be considered normal. The high amounts of sulphur and the sulphur-containing amino acids, cystine and methionine, present in nails explain the affinity of selenium for this tissue, since selenium is believed to replace sulphur in cystine

Table 7. *Distribution of selenium in human nails and urine**

Range of selenium levels in			
Nails		Urine	
No. of subjects	Se (ppm)	No. of subjects	Se (ppm)
2	0·70–0·89	6	0·015–0·034
5	0·90–1·09	4	0·035–0·054
5	1·10–1·29	4	0·055–0·074
3	1·30–1·49	2	0·075–0·094
1	1·50–1·69		
Mean . . .	1·14 ± 0·06†	Mean . . .	0·049 ± 0·006†

*Data from Hadjimarkos & Shearer (1973a).
†Mean ± SEM.

and methionine (Underwood, 1971). Obviously, nail clippings could also be used in epidemiological studies for assessing the relation between increased intake of dietary selenium and the appearance of symptoms of ill health, other than dental caries, in population groups.

Selenium in teeth

Selenium is considered a normal constituent of human teeth. The first investigations on the concentration of selenium in dental tissue were conducted by us in permanent teeth collected from individuals in Portland, Oregon (Hadjimarkos & Bonhorst, 1959). Additional studies determined the selenium content of ancient (8th–11th century BC) and modern Greek teeth (Hadjimarkos & Bonhorst, 1960) and of teeth collected in the Soviet Union (Mamedova, 1965) and in England (Nixon & Myers, 1970). The selenium content of dental enamel ranged from 0·43 to 1·6 ppm for teeth in Portland, Oregon, and from 0·21 to 2·1 ppm for teeth collected in England. The values for the selenium content of dental enamel in ancient and modern Greek teeth were only 0·13 and 0·03 ppm, respectively. These low values may account in part for the low prevalence of caries existing in Greece (Hadjimarkos, 1960b).

Surprisingly, the selenium content of enamel in deciduous teeth collected in Portland, Oregon, was 4·5 ppm, which is much higher than the levels found in the permanent teeth from the same locality, mentioned above (Hadjimarkos & Bonhorst, 1959). It may be that the placenta allows considerable amounts of dietary selenium to pass from the maternal to the foetal circulation. In the only existing study on the placental transmission of selenium in man, we reported similar levels of selenium in placental tissue and in foetal cord blood, that is, 0·18 and 0·12 ppm, respectively (Hadjimarkos, Bonhorst & Mattice, 1959).

Selenium in saliva

The presence of selenium in human saliva was reported for the first time in a recent study among school children, 11–12 years of age, in Portland, Oregon (Hadjimarkos & Shearer, 1971). All saliva samples collected from the 26 participants contained selenium ranging

from 1·1 to 5·2 ppb (b = 10^9) with a mean concentration of 3·1 ppb. Whether the level of salivary selenium increases with increased intake should be studied among subjects living in known high-selenium areas, mostly rural, and consuming locally produced foodstuffs. However, the effect of salivary selenium on caries remains to be investigated.

Selenium in human milk

In view of the existence of considerable evidence that selenium increases dental caries when consumed during the time teeth are formed, knowledge on the selenium content of human milk is of obvious importance for the breast-fed child. The first such study was conducted among lactating mothers in Portland, Oregon (Hadjimarkos, 1963). All samples of mature milk collected from 15 subjects contained selenium, with a mean concentration of 0·021 ppm. A further study was reported recently on selenium in the milk of lactating mothers living in Iowa City, Iowa, and in Athens, Greece (Hadjimarkos & Shearer, 1973b). The results are presented in Table 8 and show that the mean concentration of selenium for both groups of subjects was 0·020 ppm, the same mean level as was found in mothers from Portland, Oregon. This similarity in the selenium content of mature human milk among three groups of lactating women residing so far apart indicates that a homeostatic mechanism may be responsible for regulating the selenium content of human milk, at least at the lower levels of dietary intake. A recent report provided data on the selenium content of transitional human milk, that is, 5–10 days *post partum*. Samples were collected from lactating women living in different areas of New Zealand and the concentration of selenium was reported to range between 0·012 and 0·015 ppm (Millar & Sheppard, 1972).

Table 8. *Selenium content of milk from lactating mothers in Iowa City, USA, and Athens, Greece**

Parameter	Data from	
	Iowa City	Athens
No. of subjects	15	24
Mean age (years)	25·0 ± 0·98	27·7 ± 1·10
Mean time post partum (days)	51·1 ± 3·37	60·4 ± 8·00
Mean milk volume (ml)	22·7 ± 1·49	25·7 ± 2·14
Mean selenium level (ppm)	0·020 ± 0·001	0·020 ± 0·001

*Data from Hadjimarkos & Shearer (1973b).
Values are expressed as means ± SEM.

In contrast to human milk, the level of selenium in cows' milk varies considerably, depending upon the ration of the animal. Even within the same State, we reported a tenfold difference in the concentration of selenium in cows' milk produced in two regions of Oregon, that is, 0·005 compared with 0·058 ppm (Hadjimarkos & Bonhorst, 1961). In known seleniferous regions, the selenium content of milk was reported to range between 0·16 and 1·27 ppm, which are considered dangerous levels for human consumption (Rosenfeld & Beath, 1964).

Concluding remarks

The results of the epidemiological studies among children and of the experiments with laboratory animals, discussed above, have demonstrated that increased consumption of selenium at the time the teeth are formed increases dental caries. Experimental data indicate that the mechanism involved operates through the incorporation of selenium into the protein components of enamel and dentine, leading to inhibition of mineralization. The above studies taken together leave no doubt that the case for a direct association between levels of selenium intake and the degree of susceptibility to caries has been established. It should be pointed out, however, that in view of the multifactorial nature of dental caries it is erroneous to compare and interpret differences in the prevalence of caries seen in children living in the western and eastern parts of the United States on the basis of dietary selenium intake alone, as has been suggested recently (Allaway, 1972).

The question to be answered is what judgement should be used in deciding when an association between an environmental factor, such as selenium, and human disease becomes a biological causation. For evaluating the cause–effect significance of an association, Underwood (1970) listed nine criteria that must be utilized, although no one can be regarded as indispensable for passing judgement. The nine criteria are: consistency, strength, specificity, temporality, coherence, biological gradient, plausibility, experiment and analogy. The first five of these were originally included in the report of the Advisory Committee to the Surgeon General (1964) on Smoking and Health, and the last four were added later by Hill (1965).

The crucial evidence of a causal relationship between intake of a certain trace element and dental caries susceptibility will be provided primarily by retrospective and experimental epidemiological studies among children. Of these two approaches, the best method is to demonstrate experimentally the cause–effect relationship (Fox, Hall & Elveback, 1970). As is well known, the acceptance of water fluoridation for the prevention of dental caries was demonstrated by the undertaking of experimental epidemiological studies among children using drinking-water to which the optimum amount of fluoride was added.

However, in the case of selenium the undertaking of experimental epidemiological studies is not feasible. This is because moral and ethical considerations would preclude the deliberate addition of increased amounts of selenium to the daily diet of children during the period when their teeth are developing in order to increase the susceptibility to caries. In view of this, the judgement of causality must be based on the findings of retrospective epidemiological investigations. The independent retrospective epidemiological studies conducted among children living in Oregon, Wyoming, Montana and South Dakota, coupled with the results of the experiments with rats and monkeys, provide substantial evidence of a causal relationship between increased consumption of selenium during the period of tooth development and increased susceptibility to dental caries.

Addendum

After the completion of this review article, further evidence was presented showing that selenium increases caries in man. In an epidemiological study conducted among three population groups in the Chernovitsi region of the Soviet Union, it was found that there was a direct relationship between the degree of susceptibility to caries and the concentration of selenium in teeth collected locally which, in turn, reflected the levels of selenium in local soils (Suchkov *et al.*, *Stomatologia* 1973, **52** (2), 21).

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Toxicology: Cost/Time*

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Some type of evaluation of the toxicity of pesticides and the hazard of exposures therefrom has been carried out since the advent of pesticide use. Originally, pesticide manufacturers assumed it their moral responsibility to evaluate hazards and provide directions for safe handling and use. This developed into a legal responsibility as well. Several legislative acts have provided for pesticide regulation and all its ramifications including toxicity and safety evaluation (McCollister, 1965; US Department of Agriculture, 1968).

The Federal Insecticide, Fungicide and Rodenticide Act passed in 1947 and amended in 1959 and 1964 requires registration of all economic poisons prior to distribution in interstate commerce. Economic poisons include a wide variety of agents, pesticides among them. For registration to be obtained, data must be presented which demonstrate that the material is safe when used as directed and effective for the purpose claimed on the label. The Act also states that any residues remaining on food or feed must not exceed the established tolerance level.

The provision for tolerances is the result of the Miller Amendment in 1954 to the Food, Drug and Cosmetic Act. The original Food, Drug and Cosmetic Act, passed in 1906, provided that a food should be deemed adulterated if it contained any added poisonous or deleterious substances. The only exception was when such substances were required in production or could not be avoided in good manufacturing practice. With passage of the Miller Amendment, pesticide chemicals were officially recognized as essential to the production of food, thus providing for the establishment of tolerances in raw agricultural commodities. Tolerances are obtained by submitting to a governmental agency (formerly the FDA, currently the EPA) a petition containing extensive data covering toxicity, metabolism, residue levels, including an analytical method for determining residues on foods, and finally a reasonable basis for supporting the safety of the proposed tolerance.

Another law that has greatly influenced the establishment of tolerances for pesticides is the 1958 Food Additive Amendment to the Food, Drug and Cosmetic Act. This amendment contained the Delaney Clause, which states that no food additive shall be deemed to be safe if it is found to induce cancer when ingested by man or animals, or if it is found, after tests which are appropriate for the evaluation of the safety of the food additives, to induce cancer in man or animals. Many have interpreted this to mean that a zero tolerance must be applied to a pesticide found to produce neoplasms in animals regardless of dose, route of administration or species.

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The purpose of this presentation is to elaborate on the toxicological studies that are either required or recommended for registration of pesticides, including the establishment of a residue tolerance on food crops. In particular, the costs and time required for completion of the studies will be discussed. The values for cost and time were derived from information supplied by the following toxicology laboratories: Food and Drug Research Laboratories Inc., Dow Pharmaceuticals, Woodard Research Corporation, Mellon Institute, Hazelton Laboratories, Gulf South Research Institute, Bionetics, Diamond Shamrock Chemical Company, American Cyanamid, Eastman Kodak Company, Eli Lilly and Company, The Haskell Laboratory of E. I. DuPont de Nemours and Company, Vick Chemical Division of Richardson-Merrell, The Dow Corning Corporation, the Chemical Biology Research Laboratory of the Dow Chemical Company and Industrial Bio-Test Laboratories. Each participant provided estimates, usually as a range, for conducting the various studies described in a general protocol which was supplied. The time estimates included the time needed to conduct the experiments and prepare the reports.

In the early 1950s, toxicology requirements for registration of a pesticide were generally limited to data from single-dose oral administration and from a subacute study in which the material was fed to rats for a period of 30–90 days, with measurement of a few indicators of toxicity. Even in terms of today's inflated dollars, the cost of these studies would amount to less than $10,000.

The picture is very different today. Three factors have contributed to more rigorous and extensive toxicity testing. First the increased sensitivity of analytical methods for detecting pesticide residues has increased the emphasis and concern over the possible effects of low levels of pesticide residues heretofore undetected. Secondly, there has been an increase in the sensitivity and number of techniques for monitoring signs of toxicity, such as electron microscopy, biochemical methodology and radioisotopes and new test systems such as teratology and mutagenicity testing. Some of these developments allow measurement of different parameters of toxicity or of smaller deviations from normal in test animals, while others merely add bulk. Thirdly, an increased awareness of, and concern for, the impact of pesticides on the environment has led to more extensive studies on non-target species in the environment, whereas previously, toxicology was primarily concerned with the evaluation of hazard to man and to a species upon which the product was to be used.

The cost and time needed for completion of the acute toxicity studies required or recommended for pesticide registration are presented in Table 1. These provide data for preliminary evaluation of handling hazards as well as for characterization of the effects of acute accidental or purposeful exposures to man and to parts of the environment.

The oral LD_{50} is the most common expression of toxicity obtained. This measures the most drastic manifestation of toxicity—that of death. It is a statistically derived expression of the single dose of a material that can be expected to kill 50% of the animals treated. Various species may be used, such as rats, mice, guinea-pigs and rabbits. Along with providing numerical data, this test allows an opportunity for the observation of signs of toxicity in the surviving animals. In some cases, gross pathological and histopathological examinations are performed to reveal the target organs. Acute oral toxicity data, including the LD_{50} and description of observed effects, are recommended on at least two species of laboratory animal. The average low and high estimates of costs quoted by 14 laboratories for these studies were $796 and $1230, with a mean of $1013. A period of 4–5 weeks is required to complete the studies.

The dermal LD_{50} is also required. This is an estimate of the absorption of a material

Table 1. *Cost/time estimates for acute studies*

Test	No. of quotes	Cost		Time required for completion‡ (wk)
		Mean (M$)*	Average range† (M$)*	
Individual tests				
Oral LD_{50}	14	1·01		
Dermal LD_{50}	14	0·65		
Inhalation LC_{50}	12	0·89		
Eye irritation	14	0·26		
Skin irritation	14	0·26		
Avian oral LD_{50}	8	1·88		
Fish LC_{50}	9	0·58		
Crustacean LC_{50}	3	0·62		
Subtotal		6·15	5·16–7·11	6
Plus formulation		12·30	10·32–14·22	12
Plus 1 metabolite		18·45	15·49–21·33	18

*Thousands of dollars.
†Average of low estimates–average of high estimates.
‡If tests are run concurrently.

through the skin in acutely toxic amounts. Rabbits are the usual test animal. Other evidence of toxicity besides death is also noted. The average range quoted for such a study was $536–$758, with a mean of $647. This test requires 4–5 weeks for completion.

The inhalation LC_{50}, also required unless it is not reasonable to expect such exposures to be encountered, is the concentration expected to kill 50% of the test animals when the material is inhaled for 1 hour. This study is conducted in the rat and costs ranged from an average of $750 to $1020, mean $885. The study takes 4 weeks to complete.

In the required eye-irritation studies, the material is applied to the eyes of a rabbit and observations as to the type and severity of damage are made at various times after exposure. The effectiveness of washing the eye following application is also investigated. About 3 weeks are required to complete these studies, which range in average cost between $210 and $315, mean $262.

Skin irritation studies are also conducted on rabbits. The skin is clipped free of hair and either abraded or left intact. Evidence of local irritation, which may vary from mild redness to severe burn, is noted. The average range quoted for these studies was $217–$294, with a mean of $255. Three weeks are required for completion.

Oral LD_{50} data are required in birds as well as laboratory animals. Bobwhite quail and mallard ducks are recommended. These studies take 5–6 weeks to complete and range in average cost from $1528 to $2225, mean $1876.

Acute toxicity studies in fish are also required. The estimates had an average range of $525–$636, mean $580. Completion requires about 3 weeks.

Data on acute toxicity to other forms of aquatic organisms, such as shrimp and other crustaceans, may also be required, costing on average between $600 and $633, mean $617. Only three estimates were obtained. The studies take approximately 2 weeks.

The total cost, then, of these acute studies on a pesticide chemical would range on average from $5162 to $7111, with a mean of $6135. At least 6 weeks would be required for completion, providing the tests were run concurrently. These acute studies are also conducted

on the formulated product, and on major metabolite(s). Thus the total cost for acute toxicity studies would average $18,405, and would require 18 weeks.

The subacute toxicity studies are intended to evaluate the effects of relatively short-term repeated exposures to pesticides (Table 2). Ninety-day feeding studies are required on the parent chemical and on major metabolite(s) in at least two species. These involve the continuous administration of the test material to the animals, usually in their diets, at several dose levels. One of the species has to be a non-rodent, and dogs are most often used. The rodent species used is generally the rat. From 80 to 120 rats and from 16 to 24 dogs are used in these tests and at least three dose levels are fed. There is also an untreated control group. Observations are made regarding growth, food intake, appearance and demeanour, and mortality. Laboratory tests are conducted on urine for evidence of kidney damage and on blood for untoward effects, such as anaemia. Various organ-function tests are conducted

Table 2. *Cost/time estimates for subacute studies*

Test	No. of quotes	Cost		Time required for completion‡ (months)
		Mean (M$)*	Average range† (M$)*	
90-Day/dogs: Parent chemical	14	17·4		
Metabolite	14	17·4		
90-Day/rats: Parent chemical	14	12·8		
Metabolite	14	12·8		
21-Day dermal	11	7·0		
14-Day inhalation	10	6·1		
Reproduction: Quail	4	12·4		
Duck	4	14·8		
Total		100·7	88·7–112·3	13
Additional§		13·2		

*Thousands of dollars.
†Average of low estimates–average of high estimates.
‡Assuming studies on the parent chemical and metabolite are staggered.
§Studies on wildlife under field conditions, neurotoxicity and cataractogenic potential.

by measuring blood levels of certain substances, such as urea and protein and the activity of various enzymes. At termination of the study, selected organ weights are recorded. These usually include the heart, liver, kidney, spleen, testes and brain. Gross and microscopic examinations are conducted on approximately 30 tissues. Additional clinical studies may be conducted, such as blood and brain cholinesterase determinations in the case of organophosphate and carbamate insecticides. The objectives of these 90-day studies are to determine the nature of the effects related to ingestion of the compound and to define the minimum dosage level at which the most sensitive criterion revealed an effect and the maximum dosage at which no untoward changes were detected. The estimated cost of the dog studies ranged on average from $15,795 to $18,967, mean $17,381. The rat studies cost slightly less, averaging between $11,310 and $14,200, mean $12,765. Each study takes about 6 months for completion.

The 21-day dermal study consists of daily applications of the chemical to the skin of rabbits, with observations made for evidence of absorption through the skin in toxic

amounts. Body-weight gain and clinical and pathological parameters are evaluated. This study was estimated to cost on average between $6380 and $7520, with a mean of $6940. Completion requires 3–4 months.

A 14-day inhalation study in rats has been mentioned as sometimes desirable. It evaluates the effect of repeated exposure to pesticides via inhalation, using parameters similar to those discussed for other subacute studies. The average range of costs estimated by ten laboratories was $5590–$6640, mean $6120. The time required for completion is 3 months.

Preliminary evaluation of the effects of pesticides on reproduction in two species of birds is required. Bobwhite quail or pheasant and mallard duck are recommended. Egg production, fertility, hatchability and survival are determined. These studies take from 6 to 7 months or longer to complete. Costs average between $10,000 and $14,750, mean $12,375, for quail and between $12,500 and $17,000, mean $14,750, for mallard duck.

These subacute studies just described, including 90-day studies in two species on one metabolite, range in average costs from $88,690 to $112,290, with a mean of $100,730. At least 7 months would be required for the studies on the parent compound, assuming they were run concurrently. The feeding studies on the metabolite(s) would be conducted later and would take another 6 months. Other studies could be required, depending upon the nature and use of the compound and the results of the studies just described. For example, subacute studies on wildlife under field conditions would be necessary if indicated by LD_{50} data. Data from four sources give an average range of $2850–$3600, mean $3225. About 5 weeks are required for completion. A neurotoxicity study in chickens is required for organophosphates, costing about $1000 (one estimate). Studies for evaluation of cataractogenic potential may be indicated and could cost $9000 (one estimate). The conclusion of these additional tests would bring the average cost of subacute toxicity studies to $113,760.

Metabolism studies (Table 3) are conducted in an attempt to uncover the means by which the animal deals with the chemical. These studies include investigation of which organ(s) metabolize the material and the identification of major metabolites, absorption from the intestine, storage in various tissues, and excretion in urine and faeces. Such studies use some of the more recently developed analytical tools, notably radioisotopes and various types of chromatography and spectrometry. Metabolism studies in rats and dogs are required for pesticides. These investigations can vary considerably in extent, developing into highly complex, expensive and time-consuming efforts. The scope of the studies usually

Table 3. *Cost/time estimates for metabolism studies*

Species	No. of quotes	Cost		Time required for completion (months)
		Average range* (M$)†	Mean (M$)†	
Rat	7	9·6–20·4	15·0	3–12
Dog	7	9·2–20·3	14·7	3–12
	Total . . .	18·8–40·7	29·7	3–12‡
Man	5	17·5–24·7	21·1	1–3

*Average of low estimates–average of high estimates.
†Thousands of dollars.
‡If run concurrently.

conducted on pesticides involves a period of 3–12 months and ranges in cost on an average between $9600 and $20,433 for rats, mean $15,015, and from $9200 to $20,266 in dogs, mean $14,733.

Carefully controlled studies may also be carried out to investigate the metabolism of the pesticide directly in man and so allow for more meaningful comparisons with animal models. Subacute toxicity studies must be completed first in order to evaluate the risk involved. The experiments in man usually consist of administration of a single oral dose, followed by identification and quantitation of the parent compound and metabolites in blood, urine and faeces. The average of the range of estimates received from five laboratories was $17,500–$24,666, mean $21,080. Any time from 1 to 3 months or more may be required for completion of investigations in man.

For some pesticides, studies in man may be conducted to determine a threshold level which, if exceeded, will alter a sensitive biochemical activity. This is particularly appropriate for cholinesterase inhibitors. The chemical can be administered to human subjects at low dosages, and the blood cholinesterase activity can be measured as an indicator of physiological response, with minimum risk to the test subjects. Time and money requirements would vary considerably according to the particular design, but these studies may cost $50,000 and could take a year for completion.

Studies revealing possible effects on reproduction or foetal development are required for pesticide registration. Multigeneration reproduction studies (Table 4) are usually conducted in rats, although mice, rabbits or guinea-pigs may also be used. The animals are maintained on various doses of the test chemical throughout the study. The original parent group is allowed to produce two litters and offspring from the second litter are in turn raised as parents for the second generation, from which two litters are produced. A third generation is usually derived in the same manner. Observations are made on each breeding trial regarding fertility, survival of offspring, lactation and any evidence of abnormalities in the foetuses. These studies in rats were estimated to average in cost from $33,360 to $37,460, mean $35,410. Two years or more are required for completion. Studies in the other species mentioned cost about the same.

Table 4. *Cost/time estimates for multigeneration reproduction studies*

Species*	No. of quotes	Cost		Time required for completion (months)
		Average range† (M$)‡	Mean (M$)‡	
Rat	12	33·4–37·5	35·4	24–25
Mouse	8	22·4–27·4	24·9	24
Rabbit	4	26·5–38·8	32·6§	32–35
Guinea-pig	3	27·3–36·6	31·8	30–35

*At least one required.
†Average of low estimates–average of high estimates.
‡Thousands of dollars
§It is anticipated that studies using rabbits would cost 15–20% more than those using rats. The apparent discrepancy is attributed to the fact that laboratories submitting the highest quotes for rats did not submit estimates for rabbits.

Although the multigeneration reproduction test provides for some evaluation of teratogenic potential, separate, more specific, studies are recommended and are usually conducted (Table 5). These studies involve administration of the test material in several doses

Table 5. *Cost/time estimates for teratogenicity studies*

Species*	No. of quotes	Cost		Time required for completion (months)
		Average range† (M$)‡	Mean (M$)‡	
Rat	12	7·4–13·5	10·4	4
Mouse	9	5·6–7·5	6·5	4
Hamster	5	7·9–9·4	8·6	4
Rabbit	10	9·2–10·8	10·0§	4
Monkey	4	48·8–63·0	55·9	14–16

*One or two required.
†Average of low estimates–average of high estimates.
‡Thousands of dollars.
§It is anticipated that studies using rabbits would cost 15–20% more than those using rats. The apparent discrepancy is attributed to the fact that laboratories submitting the highest quotes for rats did not submit estimates for rabbits.

to pregnant females during the sensitive period of organogenesis. The foetuses are obtained by Caesarean section prior to the expected parturition date and are observed for evidence of abnormalities. Several species may be used, including the rat, mouse, hamster, rabbit or monkey. The cost data obtained for these studies in rats were $7373–$13,467 for the average range with a mean of $10,420. Studies in mice and hamsters are in the same price range. Generally, rabbits are a little more expensive, and studies in monkeys are 5–10 times more costly. Tests in all species except monkeys require about 4 months to complete, but at least 1 year is necessary in the case of monkeys.

The long-term toxicity studies are conducted primarily for evaluation of carcinogenic potential (Table 6). Test animals may be the rat, hamster or mouse, although the dog has

Table 6. *Cost/time estimates for chronic feeding studies*

Species*	No. of quotes	Cost		Time required for completion (months)
		Average range† (M$)‡	Mean (M$)‡	
Dog (2 yr)	12	75·9–89·2	82·6	29–30
Rat (2 yr)	13	68·5–77·1	72·8	28–29
Hamster (2 yr)	7	45·8–52·3	49·0	28–29
Mouse (18 months)	8	45·2–49·3	47·2	27–28
Total for two species (rat and dog)	...	144·4–166·3	155·3	28–30§

*Two required.
†Average of low estimates–average of high estimates.
‡Thousands of dollars.
§If run concurrently.

been used extensively in the past. Tests in two rodent species are now being suggested, lasting 2 years except for those in mice, which run for 18 months.

Residue data and subacute studies may indicate the necessity for chronic studies also on a metabolite which would double the cost. As currently conducted, these studies follow the same general design as the 90-day studies. More animals are started on the test, at least 25/sex/dose and frequently 40–100/sex/dose, to assure sufficient numbers at termination. Special attention is given to tumour formation, but otherwise clinical and pathological evaluations are similar to those in the subacute studies.

At present, there is not a firm requirement for mutagenicity studies, although they are proposed and are likely to become part of the testing programme (Table 7). The relevance

Table 7. *Cost/time estimates for mutagenicity studies*

Test	No. of quotes	Mean cost (M$)*	Time required for completion (months)
Host-mediated assay	7	1·7	2–3
Dominant lethal	9	8·1	4
Cytogenetic studies	7	7·3	4
	Total . . .	17·1†	4‡

*Thousands of dollars.
†Average range (average of low estimates–average of high estimates), M$ 15·0–19·4.
‡If run concurrently.

of the results for assessing human hazard, however, is still open to question. Three studies are recommended:

Host-mediated assay. This system involves injection of bacterial organisms into the peritoneal cavity of a mammalian host, followed by administration of the test material to the host by a different route. The bacteria are then recovered and examined for the induction of mutants. This study ranges in average cost between $1468 and $1968, mean $1718. Approximately 2–3 months are required.

Dominant lethal test. This involves mating treated male rats, usually each week for 7 weeks after treatment, with untreated females and observing mortality *in utero*. The average range of costs from nine laboratories was $7330–$9000 with a mean of $8170. The time required is approximately 4 months.

Cytogenetic studies. In vivo cytogenetic studies consist of examination of the chromosomes of treated animals and sometimes of exposed human beings for aberrations. These studies take about 4 months and cost on average from $6236 to $8380, mean $7310.

The total cost of the toxicological evaluation of a pesticide, then, would range from $306,800 to $375,480 at a minimum, with a mean of $349,800 (Table 8). The studies would take a minimum of 4 years, and more probably 6 years, to complete, depending upon how the programme was scheduled. The addition of various other studies, such as extra subacute investigations, human studies, mutagenicity studies, teratology on another species and chronic studies on a metabolite would bring the total to about $625,600. These are very likely additions and are the rule rather than the special-case exception. This is by no means

Table 8. *Summary of cost/time estimates for toxicity testing*

Test	Mean cost (M$)*
Minimum requirements	
Acute	18·4
Subacute	100·5
Metabolism, rat and dog	29·8
Reproduction, rat	35·4
Teratology, rat	10·4
Chronic feeding, rat and dog	155·3
Total . . .	349·8†
Additional tests	
Subacute	13·2
Teratology, second species	10·0
Human metabolism	21·1
Mutagenesis	17·2
Human toxicology	50·0
Chronic feeding, metabolite	155·3
Total . . .	275·8

*Thousands of dollars.
†Minimum time required for completion, 48 months.

the upper limit, either. In some instances chronic inhalation studies may be required for evaluation of carcinogenicity and these would cost in the range of $80,000–$125,000, bringing the total to almost $750,000.

The interest which the money expended for these studies would draw constitutes an additional substantial cost. Also not considered are the additional costs of administration, consultation and liaison, which may add 20% or more to the cost of a study.

This programme for obtaining acceptable toxicity data on pesticides involves large amounts of time and money. Moreover, the impact of the results is considerable, in that safety factors and tolerances are derived from them, and theoretically the health and well-being of people and their environment may depend upon the proper conduct of the tests and evaluation of the data. It is therefore appropriate to examine some of the aspects of these studies, especially regarding design and interpretation. A detailed critical evaluation of all the test methods would fill volumes, but a few points have been the subject of much discussion and bear summarizing here.

First, a brief mention is in order of how the actual test systems used have become established. Although protection of the public's health, food supply and environment from harmful amounts of pesticides has been provided for by legislation, the specific test methods employed in toxicology to provide data for safety evaluation have evolved through the years according to "the state of the art". Usually the test methods, both in their general approach and specific details, have been adopted as a result of recommendations and guidelines issued by a regulatory agency, either USDA, the FDA, or currently the EPA. They are not legislation or "law", but although allegedly this is not the intent, they become *de facto* minimum standards, and when another experimental approach seems equally or more appropriate, it must be undertaken in addition to, and not instead of, the official decree. Because of this shift from recommendation to required standard, newly proposed official guidelines should be, and usually are, challenged if considered inordinately restrictive,

scientifically unsound or impractical, by experienced members of the toxicological community. Unfortunately, the track record of such challenges has been very poor.

Several factors in the design and conduct of repeated exposure experiments have prompted considerable discussion. First there is the selection of species of animal to be used. There are a few prerequisites regarding this. There are practical considerations, such as size, availability, ease of maintenance and handling and short lifespan. Good background information regarding normal clinical values, mortality, tumour incidence and other characteristics is absolutely essential. The ideal choice is the species that metabolizes a given chemical in the same manner as does man.

The scheme for determining this most appropriate test species has been described by various groups (*Food and Cosmetics Toxicology*, 1969; Food Protection Committee, 1970; Frazer, 1970; Frazer & Sharratt, 1969). Generally, it involves preliminary subacute studies in several species, from which data regarding both toxicity and metabolism can be obtained. Then, if not contraindicated by high toxicity, limited tests in man are undertaken for determination of metabolites in blood, urine and faeces. At this point it may be possible to make a reasonably good choice of species for longer-term studies. This is an extreme oversimplification of the problem for most cases, however. Numerous species may be tested, still without good correlative data. In the case of coumarin, ten species were examined before one was found that was similar to man in its metabolism of this compound (*Food and Cosmetics Toxicology*, 1970; Kaighen & Williams, 1969; Shilling, Crampton & Longland, 1969). If the species were an exotic one, difficult to handle or without adequate background information, the disadvantages could well override the advantage of an apparent metabolic similarity. The value of the information gained must be balanced against opposing factors.

The species commonly used in repeated toxicity testing have various advantages and drawbacks. The rat is the most widely used, for both its practicality and physiology. There is general agreement as to the value of studies in rats (Edson, Noakes & Sanderson, 1968; Frazer, 1969). The dog is the non-rodent species of choice for subacute testing, but its long lifespan renders it inappropriate for carcinogenic evaluations. This raises the point of the rationale of 2-year feeding studies in dogs. These studies are intended for evaluation of carcinogenicity and extend for the lifetime of other species. Furthermore, Weil & McCollister (1963), in comparisons of 21 long-term rat and dog studies, showed that dogs were no more sensitive than rats regarding other indications of toxicity. Therefore, it would seem logical to conduct studies of shorter duration in dogs since it would be much less expensive and just as valuable, as long as attempts are not made to ascertain carcinogenicity. The dog is susceptible to bladder carcinomas induced by certain aromatic amines, where other species have proved refractory (FDA Advisory Committee on Protocols for Safety Evaluation, 1971). In these cases, the 7-year or more experimental period is warranted. There are indications that the hamster may prove to be a satisfactory substitute.

Mice are used in chronic studies, especially those for the evaluation of carcinogenic potential. They are, however, very susceptible to a wide variety of spontaneous and induced tumours and their suitability for a reasonable carcinogenic investigation has been questioned (Grasso & Crampton, 1972).

Monkeys have received considerable attention as test animals. There is no evidence, however, that they are closer to man with regard to metabolic patterns than are rats or dogs (Committee on Problems of Drug Safety, 1969; Frazer, 1969; Noel, 1970). In fact, being herbivores, evolutionary adaptive mechanisms have no doubt left them quite unsuitable for evaluating the toxicity of many agents.

Various other animals have been used in special cases. The use of neonatal mice and hamsters has been in vogue in the last few years for carcinogenicity testing and has met with criticism, as the interpretation of results is most difficult (*Food and Cosmetics Toxicology*, 1968; Task Force on Research Planning in Environmental Health Science, 1970).

Earlier in this paper, mention was made of the standard clinical tests that are determined in subacute and chronic feeding studies. A few of these will be discussed in more detail at this point. Urine analyses are routinely conducted, although results are almost always normal, even in cases of morphological renal damage. Blood urea nitrogen, the most common determination for evaluation of renal function, is likewise very insensitive, normal levels being found even with severe renal damage (Sharratt, 1970). Measurement of bromsulphthalein (BSP) dye retention is a liver-function test routinely conducted in dogs. However, up to 50% of the functional capacity of the liver must be impaired before the dye test will indicate the damage (Cornish, 1971). There seems to be little scientific basis, then, for routinely conducting these tests. Rowe, Wolf, Weil & Smyth (1959) and Weil & McCollister (1963) compiled and compared data from a number of short- and long-term feeding studies and found, among other things, that the most efficient criteria for defining the lowest dosage level that produces an effect are body-weight gain, liver and kidney weights and pathological alterations in the liver and kidney. This is not to suggest that these should be the only criteria examined, but that certain clinical studies should be included only when indicated and not run routinely according to some "recipe". Furthermore, even if omission of these tests resulted in missing a change (however unlikely), the effect would be detected by other, more sensitive criteria, so there would be no loss of information. Weil & McCollister (1963) also found that results of 90-day studies are excellent predictors of the outcome of 2-year trials. Other investigators share the belief that studies of a few months duration will reveal the nature of important toxic effects, except for carcinogenicity (Paget, 1963 & 1968). Thus long-term studies could truly concentrate on the evaluation of carcinogenic potential, rather than be cluttered up with meaningless and repetitious data.

The selection of dosage levels administered in repeated dose studies is an important decision. Doses for subacute studies may be estimated from LD_{50} data, but this is often not very successful. Weil, Woodside, Bernard & Carpenter (1969) have shown short-term (7-day) tests to be much more efficient in predicting optimal dose ranges for longer term studies. In well-designed toxicological investigations, a range of doses is administered so that dose-related effects will be evident and a safe threshold dose can be calculated. The establishment of this dose-response relationship is "fundamental to all toxicological endeavour" (Golberg, 1971).

There are those in the field who advocate the administration of massive doses, presumably in an attempt to maximize the likelihood of toxic manifestations. One wonders if this might be partly due to an aversion to negative data. Striking results attract much more attention, both from certain scientific publications and from the lay media. However, the effects from excessive doses may simply be evidence of abnormal metabolism as a result of overloading the system, and are not necessarily reflections of inherent toxicity or characteristic of changes occurring at lower doses (Food Protection Committee, 1959). It is likely in many instances that normal metabolism could handle the chemical in more reasonable quantities (*idem*, 1970). Data obtained from animals given massive, unrealistic doses are especially inappropriate for assessing hazard in the light of the current emphasis on the effects of exposure to very low levels of pesticides over extended periods, perhaps a lifetime.

The practice of administering massive doses really becomes a serious problem when a

zero tolerance is applied on the basis of the results. This is true in the case of suspected carcinogens, as provided by the Delaney Clause, which states that no level of an additive is safe if it is found to induce cancer in man or animals by appropriate test procedures. This sounds like a noble pronouncement, until one realizes that the qualification in the clause relating to appropriateness is often ignored in designing experiments and interpreting results. A definition of appropriateness is definitely in order, so that our food supply will not be jeopardized by results from poorly designed, irrelevant toxicity studies.

Although not included in the Delaney Clause, the concept of "zero tolerance" has been extended by some people to cover agents that have been shown to be teratogens and mutagens, regardless of dosage, in animal studies. Various factors which may influence foetal development are notorious. Golberg (1971) has summarized these, which include transport of mice by air on days 12 and 13 of pregnancy, fasting at a critical stage or a diet of raisins for one day, along with other stress factors. Interpretation of results from mutagenicity studies is in an extremely naïve stage, with really no understanding of how the findings might apply to man. This is not to suggest that these new test systems be abandoned or ignored. However, before high-impact decisions are reached on the basis of the results, more progress should be made in evaluating the significance of findings, including consideration of dose–response relationships.

Since many of the problems in toxicity studies arise in the interpretation of data rather than in methodology, a few more words on this subject are in order here. Reverence is often made to "the level of no effect" defined by toxicity investigations, implying that thousands of dollars and sometimes years of effort have been reduced to a single number. Indeed the significance of this expression should not be minimized; not only is it of practical importance in that it is used in calculations of safety factors and tolerances, but behind it lies the primary concept in toxicology—that there is a safe level of virtually any material, below which "there is practical certainty that injury will not result . . ."; this is safety, as defined by the Food Protection Committee (1959). Arriving at a no-effect level for a given compound requires judgement based on broad experience in toxicology. The concept of the term is a dynamic one, which has to be re-evaluated with each new, more sensitive test system. One of the major interpretative problems in toxicology is that of evaluating the significance of subtle changes detected by various criteria. How these are interpreted has a definite influence on the final no-effect level defined. One of the most obvious examples of this kind of change is that of increased liver weights. Chapters have been written regarding the significance of this, and there is still some lack of agreement, although the general consensus seems to be that, in the absence of functional and morphological changes in the liver, this effect is without toxicological significance.

There are other examples of interpretative problems, but space does not permit elaboration. In general though, dilemmas still exist in distinguishing between "response and injury; no harmful effect and no detectable effect; adaptation and damage; permanent and transient damage; an acceptable and an unacceptable positive finding . . ." (Frazer, 1969).

Along with the enormous task of solving some of the interpretative problems just mentioned, there are other situations that warrant high-priority attention. More consideration should be given to naturally occurring toxins; their contribution to the exposure level of carcinogens, mutagens and teratogens should be explored. The benefits of pesticides should be weighed very carefully against the risk involved. The production of food without suitable pesticides is a frightening prospect and hasty decisions should be avoided except in a case of a real public health hazard.

Conclusions

It is very clear from this information that obtaining toxicity data on pesticides for evaluation of safety is an expensive, time-consuming procedure. Moreover, the impact of the results is considerable in that tolerances are derived from them and theoretically the health and well-being of people and their environment may depend upon the proper conduct of the test and evaluation of the data.

Often overlooked are the conservative judgements made in establishing a safe level for a pesticide. After a "no-effect" level of a pesticide for laboratory animals is established, a safety factor of 100 is frequently used in extrapolating to a safe level of intake for man, a level commonly designated as the ADI, the Acceptable Daily Intake. A tolerance level for a given pesticide established by regulation takes into consideration not only the projected safe level, but also the actual residue levels that result from the prescribed use in various commodities and the consumption patterns of the individual commodities.

Although some of the tests in question may be scientifically valid in themselves, they are inappropriately designed and/or interpreted to enhance the establishment of safety. Many procedures in toxicological testing are not only costly but redundant. Even more disturbing is the fact that many of the redundant procedures are less sensitive than others for detecting an untoward effect. These low-yield, high-cost procedures are performed to satisfy whims rather than to provide data for safety evaluation.

Currently, these policies are at the expense of the pesticide manufacturers and therefore ultimately the consumer. Hopefully, they will not render the pesticide business so prohibitively expensive and the business risk so great that the food supply of the nation and the world will suffer.

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BOOK REVIEWS

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 43. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1972. pp. vii + 149. DM 44.80.

This useful series continues with a volume devoted mainly to an examination of the fate of some important pesticides in animals, crops and soils. The fact that the four contributions originated from the USA, England, Holland and Canada, illustrates again the international popularity of this series, which has much to offer to readers from a wide range of biological disciplines.

The first article relates to the biochemical degradation of organophosphorus insecticides in animals and plants, a subject of great toxicological significance since a given biochemical transformation may give rise to a toxic metabolite or may serve to activate or inactivate the parent compound. Adequate attention is given not only to the functions of relevant enzymes but also to their distribution, the activity of a particular biological catalyst being an important parameter in determining whether a given compound is produced in quantities large enough for its individual toxicological properties to become effective. The need for further work is stressed, especially in cases where more than one enzyme combines with the same site on a pesticide molecule. In such a situation, the establishment of relative reaction rates is an essential step in the development of selective chemicals for influencing specific reactions.

The second paper is concerned essentially with the degradation of the related herbicides, dichlobenil and chlorthiamid, in plants, soils and animals. The two compounds are considered together, since their chemical similarity suggests many parallel metabolic steps in their breakdown. A realistic approach is maintained by relating the results of laboratory studies to actual field conditions wherever possible. It appears that the metabolic transformations undergone by these herbicides give rise to compounds which are not likely to accumulate in animal tissues.

The fate of dichlobenil in plants and soils is examined more deeply in the following article, the roles of sorption, translocation, evaporation and degradation in determining the availability of the herbicide being explored in detail. In soil the herbicide is degraded microbiologically, the principal metabolite being 2,6-dichlorobenzamide (BAM). The herbicide itself is absorbed by plants and distributed throughout the organism, evaporation and degradation being competitive processes. BAM is also absorbed by the plant and this, together with its hydroxy metabolites, forms the main herbicide residue in plant tissues. Herbicidal activity is directly associated with dichlobenil, but it is stressed that BAM is responsible for at least one side-effect in treated crops (leaf margin chlorosis), and the analysis of the mode of action of dichlobenil itself may be complicated by the activities of its degradation products.

In keeping with one traditional aspect of these reviews—the monitoring of current improvements in pesticide detection—the final paper in this volume examines the technique of thin-layer chromatographic-enzyme inhibition (TLC-EI). Since organophosphorus and

carbamate pesticides are potent enzyme inhibitors, such a method lends itself to the sensitive detection and determination of such residues, and has the notable advantage of being capable of rapid and simultaneous screening of pesticide residues in crops. The technique is subjected to careful scrutiny by the author, and its shortcomings are well documented as well as its merits. Those intending to use this technique should find this chapter of considerable value, both from the theoretical standpoint and as a starting-point for laboratory work.

Epidemiology of DDT. Edited by J. E. Davies and W. F. Edmundson. Futura Publishing Company, New York, 1972. pp. ix + 157. $14.95.

Much has been said about organochlorine pesticides and their ubiquitous occurrence in the environment since their introduction in the 1940s. This book is essentially a collection of papers (most of which have already been published in various journals) derived from a community study of pesticides carried out in Dade County, Florida.

The first chapter outlines the aims and general background of this study of the chemical epidemiology of DDT, defining chemical epidemiology as "a description of the contamination of people without specific reference to the disease-producing potential" of the chemical. The object is therefore to establish the frequency and distribution of the chemical in man. Dade County is a sub-tropical marine environment with lush vegetation in which continuous public and private pest control measures are carried out. It is recognized that because of the high levels of pesticide usage in this area, the findings are not necessarily applicable to other regions, but there is clearly much common ground between this work and studies made elsewhere. A second useful introductory chapter provides a background of the usage, toxicity, pharmacology and metabolism of p,p'-DDT. The methods of analysis used for DDT and its metabolites in fat, blood, and urine are given in a series of appendices; these basically gas-chromatographic methods were developed to meet the essential criteria of simplicity, speed and reproducibility demanded by studies of this kind.

An initial study of fat samples taken at autopsy from subjects experiencing accidental or violent deaths showed variations in DDT and DDE levels which were reflected in DDE blood levels in the general population. Further comparisons of autopsy and biopsy samples of fat tissue with blood levels of DDT and DDE showed that DDE blood levels reflected DDT levels in body fat very well. In view of the ease of blood sampling compared with fat biopsy, blood DDE thus offered a useful means of monitoring DDT and DDE in epidemiological studies. Studies of both blood and adipose tissue from the general population indicated that DDT residues increase in children up to the age of about 10 years and then level off and that residues are higher in non-white subjects, the lowest residues being found in white females aged 0–5 years. In formulators, sprayers and agricultural workers, DDE levels tended to reflect the years and frequency of exposure, whereas DDT levels were transient, reflecting only recent exposure.

The importance of other factors, particularly those affecting microsomal-enzyme activity, on the tissue accumulation of DDT residues was underlined by the group's findings of remarkably low or negligible residue levels in patients, including farm workers, undergoing treatment with anticonvulsant drugs such as phenytoin and phenobarbitone.

Transplacental passage of DDT has been demonstrated, but the racial differences apparent in the DDE levels of the maternal blood were not reflected in the foetal blood levels. The presence of measurable levels of DDT and DDE in the cord blood, amniotic fluid and foetus

and preliminary indications of some correlation between high foetal DDE levels and prematurity has led to speculation on the possible release of stored DDT if some stress condition in mother or foetus leads to rapid fat-mobilization and on the theoretical possibility that microsomal-enzyme stimulation by the released pesticide could promote premature labour by reducing available progesterone. However, as the authors of this brief but interesting chapter point out, much more work will have to be done before any conclusions can be drawn on these possibilities.

Having established the overall pattern of DDT residues in various sectors of the population, the group questioned the sources of contamination. The major source of DDT residues is generally accepted as being the diet in persons who are not occupationally exposed to DDT, but there is a strong suggestion in this volume that, in Dade County at least, environmental factors also play an important part. Dietary considerations and, to some extent, genetic factors cannot be dismissed, but socio-economic factors appear also to be involved. The importance of environmental factors was demonstrated in a small but unusual experiment in which two kittens from the same litter were placed in two different households. In one household, all the family had high blood levels of DDE, while in the second the levels were relatively low. The cats were both fed the same commercial cat food, but when their blood was analysed for DDE after a few months the cat in the former household was found to have a markedly increased DDE level, while that of the cat in the low-residue household remained low and stable.

It is emphasized that the primary objective of this community study was not the correlation of disease with DDT residues but the assessment of the contamination of man, and the development of an appropriate methodology for this type of problem. The presentation of a series of individual papers inevitably results in some lack of fluency, but the book as a whole makes an interesting contribution to the literature on DDT residues and the demography of their contamination of man.

Mercury: A History of Quicksilver. By L. J. Goldwater. York Press, Baltimore, Md, 1972. pp. xi + 318. $15.

Methylmercury. A Review of Health Hazards and Side Effects Associated with the Emission of Mercury Compounds into Natural Systems. By G. Löfroth. Ecological Research Committee Bulletin no. 4. 2nd ed. Swedish Natural Science Research Council, Stockholm, 1970. pp. 59. 10 kr.

The author of the first book cited above worked for many years on the occupational problems of mercury in the hat industry and on the absorption and excretion of mercury in man, before turning his attention "in retirement" to the part played by mercury in human history. The result is a collection of fascinating and exhaustively researched snippets of information, obviously written by someone with a great love for his subject. The book is divided into two parts, the first dealing with mercury's occurrence, mining and extraction, and with its role in trading, in the occult arts and in the development of chemistry and technology. The second part is devoted to the importance of mercury in medicine throughout the ages and to associated subjects such as its toxicology and pharmacology, the levels normally found in man and methods used for its analysis.

It is interesting to read of the elevated status mercury attained in very early times. The entire structure of alchemy was based on the "tria prima" of mercury, sulphur and salt, and mercury was credited with magical powers of life, growth and reproduction which

would naturally culminate in the formation of the perfect metal, gold. Mercury in the form of cinnabar was used for painting the interiors of tombs and other sacred objects, and may even have adorned the Great Wall of China. In more recent times it enabled Boyle to formulate his famous law and helped in the discovery of oxygen, leading to an understanding of combustion and respiration and to the downfall of the phlogiston theory.

In medicine, mercury's most important application, spanning more than 400 years, has been in the treatment of syphilis, although the author questions whether its distinguished reputation in this respect was in fact deserved. Doubt is also cast on the efficacy of mercurial ointments for the skin and on other relatively recent uses of mercury compounds, including their incorporation as antibacterials into throat pastilles and toothbrush bristles. According to one (apocryphal?) story, mercury treatment for syphilis was the origin of its use in the felt-hat industry. It is said to have been the practice for workers to urinate on camel hair to accelerate the felting process, and one worker undergoing mercury treatment for a venereal disease achieved far greater success than his colleagues! On a more sombre note, it is horrific to read that the average life expectancy of Peruvian mercury miners was at one time only about 6 months.

The book was apparently completed too early for it to include much of the data on mercury toxicology, pharmacology and occurrence in foodstuffs and in man that has flooded the literature in the last few years. Indeed, surprisingly few of the references chosen for citation on these subjects are from the last decade, although in view of the historical theme of the book this is perhaps understandable. Considered as a whole this book may be thoroughly recommended as background reading, entertaining and instructive even for those not directly concerned with the current problems raised by the metal.

The second publication listed above complements the first to some extent, since it is devoted entirely to a toxicological appraisal of environmental contamination by methylmercury. Written by the head of the Swedish Working Group on Environmental Toxicology, this report was originally published in March 1969 and was updated in September 1970 with a 9-page addendum covering some 46 additional references. In contrast to the first book, practically all of the total 157 references cited are from the last decade. Although written with particular reference to the situation in Sweden and intended specifically as a critique of the official Swedish tolerance level of 1 ppm for mercury in fish, the publication is of much wider relevance.

The first topic to be discussed is methylmercury poisoning in man, including the Minamata incident, pathological and clinical features of severe methylmercury poisoning and the long-term effects of brain-cell damage. The effects of methylmercury seed dressings on non-target organisms are documented, with evidence of the decline in mercury levels in food following the Swedish 1966 ban on the use of such materials. This leads on to a discussion of methylmercury in Swedish fish, its origins and the reasons behind the official tolerance level. Further sections deal with the genetic and metabolic effects of methylmercury, animal toxicity data and occupational exposure to alkylmercury compounds. The correlation between methylmercury intake and mercury concentration in the body, the accumulation of mercury in the foetus and the biological half-life of methylmercury are considered in connexion with the levels of mercury found in man.

The author concludes that while agricultural use of methylmercury is highly undesirable, air or water pollution with any mercury compound may pose even greater problems, in view of the possibility of biological methylation. Some deficiencies in available data noted in the 1969 conclusion had been rectified by the time the 1970 addendum was written.

This addendum records that in 1970 the official recommendation on fish consumption was modified to make the point that fish containing 0·2–1·0 ppm mercury should not be eaten more often than once a week, and it is interesting to speculate whether the initial publication of the report brought this about.

The text suffers from occasional linguistic lapses, but on the whole the book is to be praised for its lucid presentation of recent data on methylmercury and for its assessment of the potential hazards from this and other mercury compounds in the environment.

Harry's Cosmeticology. The Principles and Practice of Modern Cosmetics. Vol. 1. By R. G. Harry. 6th ed. Revised by J. B. Wilkinson. Leonard Hill Books, Aylesbury, Bucks., 1973. pp. xxiv + 824. £13.50.

The familiarity of "Harry", particularly to research and development workers in the cosmetics and toilet preparations field, has been acknowledged by an official change in title to *Harry's Cosmeticology* for this, the sixth edition of *The Principles and Practice of Modern Cosmetics, Volume 1.*

This acknowledgement of "Harry" is a just tribute to the author who aimed, with marked success, to provide a book that was not a mere formulary but furnished a sound basis for research and development by explaining and illustrating the principles behind the formulation of cosmetics. "Harry" can justifiably be said to be concerned with "deviations from the norm of skin, hair and teeth occurring or tending to occur under the normal daily stresses of work, wear, dirt, exposure and climate, and not excluding psychological stress". Thus although this is not, and indeed does not aim to be, a textbook of physiology, every effort has been made to provide a guide for further study by including references to leading texts and review articles. The practice of including classic formulae in addition to modern ones has been continued, as it was felt that they illustrate the essential elements of the product properties and may indeed still be appropriate in some circumstances.

In this latest edition, all chapters have been revised and updated to include developments that have taken place since the fifth edition was published in 1962. The intervening decade has seen a marked scientific advance in all aspects of cosmetics development, but particularly in relation to teeth. Progress in this field has benefited from considerable research activity in both industrial and academic circles. In consequence, the chapter entitled "The Tooth and Oral Health", has been substantially rewritten. In the pure cosmetics field, there has been less to record and the commercial advances of the past few years have not been based on major scientific improvements. There are signs, however, that a much-needed revival in work on the physiology and biochemistry of the normal skin is taking place and consequently the opening chapters (nos 1, 2 and 3) have been rewritten from a more biochemical point of view, so that the "day-to-day skin aberrations which are not sufficiently serious to merit medical attention" can be better appreciated.

New chapters have been included on special products developed with the needs of adolescents, the particular problems of usage in extreme climates and the growing demand for means of hair straightening and skin bleaching very much in mind. The chapter on pressurized packs has also been extensively modified and expanded in line with commercial and technical advances. A new chapter has been added on general principles of packaging. Finally, for ease of reference, an appendix has been included, listing the chemical descriptions and suppliers of proprietary materials cited in this volume.

Up-to-date revised versions of standard and reliable reference books are always welcome. This is particularly true of "Harry".

Foreign Compound Metabolism in Mammals. Vol. 2. Senior Reporter D. E. Hathway. The Chemical Society, London, 1972. pp. xv + 513. £11.

This book, the second in a series to be published in future biennially, reviews the literature published in 1970 and 1971. Volume 1, spanning the literature from 1960 to 1969, was reviewed in an earlier issue (*Cited in F.C.T.* 1972, **10,** 693).

Basically, Volume 2 is an extension of the preceding volume and utilizes the same somewhat cluttered format. However, the addition of a compound index, which also covers Volume 1, is a great improvement, although ideally a subject index would also be advantageous. In all, the book contains seven chapters relating to various aspects of the interaction and biotransformation of xenobiotics in mammalian systems.

The first two chapters are devoted to the preparation and use of radio-isotopes in metabolic studies. Another chapter provides extensive coverage of the biotransformation of many classes of drugs, food additives, pesticides and other economically important materials, as well as of a variety of carcinogens and toxins. As in the rest of the book, this part of the text is well supported with structural formulae and metabolic pathways. The actual mechanisms of xenobiotic transformations are considered in detail in the fourth chapter, special attention being paid to cytochrome *P*-450 and other components of the microsomal electron-transport chain, while chapter 5 gives details of species, sex and strain differences that often give rise to qualitative as well as quantitative differences in xenobiotic metabolism.

Many workers are becoming increasingly aware of the importance of kinetic studies of drug absorption and distribution. Chapter 6 establishes the general principles of drug absorption and bioavailability, illustrating the theory with many appropriate examples. The seventh and final chapter considers another subject of current concern, namely the interactions between various drugs and other foreign compounds. The examples considered include barbiturates, anticoagulants and antidepressants.

This book provides a comprehensive survey of the literature published during this 2-year period, providing the reader is prepared to search for his information. However, at £11 for each volume, this series may not attract many private buyers.

Current Topics in Biochemistry. National Institutes of Health Lectures in Biomedical Sciences. Edited by C. B. Anfinsen, R. F. Goldberger and A. N. Schechter. Academic Press, New York, 1972. pp. x + 255. £3.

This volume marks the first publication of some contributions to a well-established series of lectures held twice a year at the National Institutes of Health (NIH) at Bethesda, Md. The lectures, given by the staff of NIH, form part of an advanced teaching programme, including a series of seminars as well as formal lectures, and are aimed primarily at young physicians being trained in biomedical research at the Institute. The lectures in this volume, and in others which are planned, were intended to record not only the authors' own contributions to the field of study under consideration, but also to give a broad review of the subject.

Inevitably the seven topics covered in this first volume form a rather disjointed selection representing, as they do, a cross-section of the research interests of this internationally renowned group of institutes. The book includes chapters on topics of immediate interest and active enquiry, such as cyclic AMP, cell surface receptor sites, muscle protein and the mechanism of muscle contraction and RNA-dependent DNA polymerases, as well as interesting expositions on membrane structure and function, collagen and the genetics of abnormal lipid metabolism.

Although volume 2 of this series will be specifically related to current problems of oncology, the first two chapters in the present volume also touch on this field. R. O. Brady's chapter on abnormal lipid metabolism deals initially with a number of inherited diseases shown to result from the lack of a specific lipid catabolic enzyme and shows how this work has led to investigations of altered lipid metabolism in neoplastic cells, particularly with regard to membrane glycolipids. The reference to cell transformation by RNA-viruses in this chapter is followed up in the second chapter by E. M. Scolnick's consideration of the RNA-dependent DNA-polymerases (the so-called "reverse transcriptases") of RNA-containing tumour viruses. An account of the biology of these viruses is presented, particularly with respect to mammalian systems, and this is followed by a summary of current knowledge on the mechanism of integration of the virion into the host cell. The gaps in current knowledge are stressed, especially in connexion with the final step in the initiation of new DNA synthesis.

With the vast literature already in existence on cyclic AMP, any informed review must be welcomed. The chapter by I. H. Pastan deals with some selected areas of this field, including a description of the general Sutherland model. It also considers adenylate cyclase, cyclic AMP phosphodiesterase, and the mechanism of action of cyclic AMP. This section is completed by some detailed biochemistry of the effect of cyclic AMP on bacterial systems.

Cyclic AMP plays a central role in the action of hormones on cells, and therefore receives considerable attention from M. Rodbell in his chapter on cell-surface receptor sites. After pointing out the enormous possible complexities of biological membranes made up of lipids, proteins and carbohydrates, he develops his argument of information transfer across membranes in terms of interaction between these basic units. He explains in detail the action of the hormone, glucagon, stressing gaps in our knowledge such as the nature of the interaction between hormone and its discriminator (is phospholipid involved?), and the interaction between discriminator and adenylate cyclase (the so-called transducer function).

Transfer of molecules across membranes is dealt with by S. Roseman. He discusses the use of bacteria in this field, a development which was particularly valuable because it permitted the application of genetic manipulation to this branch of biochemistry. He describes the enzyme system that phosphorylates sugars, by a complex sequence of phosphoryl-group transfers, and presents evidence to show that this system is involved in the transport of sugars across bacterial membranes.

The volume is completed by two chapters on protein topics. A general review on collagen is presented by K. A. Piez, who provides interesting information on amino-acid sequences and the types of cross-linking, with particular stress on species variations. The nature of the cross-bridging within the complex muscle protein is of vital importance to an understanding of muscle contraction, a point discussed at some length in W. F. Harrington's contribution. The structure and stability of striated muscle protein is well reviewed here.

In all, this volume is a useful addition to the review literature.

Biochemical Responses to Environmental Stress. Edited by I. A. Bernstein. Plenum Press, New York, 1971. pp. xii + 153. £3.75.

The ability of living organisms to adapt to a changing chemical and physical environment is an area of ecological research that is receiving increasing attention. It provided the basis for a symposium organized by various divisions of the American Chemical Society and held in Chicago in September 1970. Eight papers presented to this symposium by sixteen contributors provided specific illustrations of the role of homeostatic control in the response of microbial and mammalian systems to environmental stress and are reproduced in this volume.

Four of these contributions deal with microbial systems and are concerned with the effects of novel substrates and with adaptation to extremes of temperature, pH and exposure to irradiation. R. P. Mortlock and W. A. Wood describe the genetic and enzymatic ability of *Aerobacter aerogenes* to metabolize certain 5-carbon sugars not commonly found in nature. The remarkable ability of *Escherichia coli* to regulate the lipid composition of the cell membrane in the face of changing conditions of temperature and changes in fatty acid substrates is discussed by S. J. Wakil and M. Esfahani. Microbial adaptation to extremes of temperature and pH and the ability of spore-forming bacteria to withstand irradiation by ultraviolet light form the subjects of papers by T. D. Brock, and by J. E. Donnellan, Jr., and R. S. Stafford, respectively.

The response of animals, particularly the rat, to chemical and physical stress is described in the other four papers. D. F. Scott and his colleagues discuss various factors affecting the diurnal variation in the activity of tyrosine aminotransferase in the rat, while the other topics range from the patterns of enzymatic response to various transplantable tumours (by C. Wu and J. M. Bauer) to the biochemical aspects of acclimatization to a cold environment (R. E. Beyer) and the effect of DDT and its derivatives on hepatic microsomal drug-metabolizing enzymes (D. Kupfer). Finally, a fairly detailed subject index has been added.

This book attempts to provide some useful, albeit limited, information on this vastly expanding field of environmental research. In this it achieves a qualified measure of success, a result not infrequently encountered in the publication of symposium papers.

Laboratory Techniques in Biochemistry and Molecular Biology. Vol. 3. Edited by T. S. Work and E. Work. North-Holland Publishing Company, Amsterdam, 1972. pp. viii + 610. Dfl. 95.

The phenomenal advances in the past two decades in molecular biochemistry have been made possible by the development of many new and highly sophisticated laboratory techniques. The intricacies in methodology doubtless familiar to those actively involved in this field are bewildering and frustrating to the novice. This series attempts to fulfil a long-felt need for laboratory manuals which clearly spell out experimental procedures to be followed, and this particular volume is divided into two parts dealing, first, with the determination of sequences in RNA and, secondly, with techniques in lipid analysis.

The section on methods for the determination of sequences in RNA, by G. G. Brownlee, includes a short introduction on historical aspects, the general approach to sequence determination and nomenclature. The author has attempted, with commendable success, to give the experimental details necessary for the successful execution of methods for the

determination of the sequence of non-radioactive RNA, for high-voltage paper electrophoresis, for end-group analysis of RNA and for *in vitro* studies using radioactive (^{32}P-labelled) RNA.

The section on lipid analysis, by M. Kates, includes an extremely useful introduction dealing with the definition and classification of lipids. The analytical procedures described for lipid extraction and separation and for identification of individual lipids and lipid moieties are uniformly excellent.

Both parts are adequately and separately indexed and include, in addition to relevant references, a very useful section on suppliers of chemicals and equipment. This book should prove a valuable laboratory work manual for biochemists.

Methods in Morbid Anatomy. By R. R. Wilson. Butterworths, London, 1972. pp. xi + 156. £4.25.

The examination of tissues and organs *post mortem* is an important step in the pathological diagnosis of disease processes. Much may be missed if the dissection of tissues is haphazard or carelessly conducted. The art of systematic dissection for displaying morbid processes to their best advantage can only be learned in the post-mortem room, and although there is no substitute for this gruelling discipline the pathologist is always glad to learn from the experiences of others.

In this book, a pathologist of long standing sets out the methods of approach to autopsy examinations that he has found best in the practice of his profession. He has confined himself only to methodology, leaving aside descriptions and interpretations of the abnormal. His instructions are clear and readily understandable, although one may deplore some lapses into colloquialism. The diagrams are especially good and provide excellent illustrations of a number of techniques which would otherwise be difficult to describe briefly. An important section of the book deals with the microscope and photomicrography and contains many useful hints and explanations.

Few would find fault with the techniques the author recommends. They are practical and logical and although not necessarily easy should not present any major difficulties. The young pathologist will find in these descriptions a valuable guide through many an awkward autopsy, while his more experienced colleague will be only too glad to learn of approaches and techniques which are different from, and perhaps better than, the ones he has painstakingly acquired over the years.

The Testing of Chemicals for Carcinogenicity–Mutagenicity–Teratogenicity. Health Protection Branch, Ministry of National Health and Welfare, Canada, 1973. pp. vi + 185. Available free on request from Information Canada, Ottawa, Canada.

This little book is the culmination of many months of devoted effort by three expert committees, seeking to compile an account of the present state of the art in the areas of testing for carcinogenicity, mutagenicity and teratogenicity. Currently, there are no fields of toxicology in greater need of clarification than these so-called "long term irreversible effects". In this book the reader will find critical, balanced and practical discussions of the theoretical background, the current practice and the prospects for future development in the areas under consideration. What a welcome change from the axe-grinding, 'hard sell',

public-relations pronouncements that have characterized so much of the writing on these subjects! What a relief to find not only sections on interpretation and evaluation, but even one on "Risk Acceptability". The following quotation affords a fair measure of the common-sense approach that imbues the discussion of acceptability of hazard:

> "Decisions on the regulation of chemicals can never be divorced completely from the milieu in which they are made—socio-economic, cultural, legal and philosophical considerations are often important modifiers of the type and speed of action which is deemed appropriate (e.g. on the use of tobacco). Scientific assessments of risk and loss and to some extent of benefit, can and should avoid the premature introduction of these factors. In particular, they should take no account of the notoriety of a compound, and should resist pressures to categorise chemicals ("X is known teratogen" or "Y is generally recognised as safe") and thus bias the assessment process.
> Evaluations of risk acceptability are reviewed, and if necessary changed, when further research information becomes available. This is an indication of strength, flexibility and integrity, not of weakness and vacillation."

From the regulated industries that produce drugs, pesticides, food additives and other chemical-agents arises the constant cry "Tell us what to do and we'll do it". This is the classical fallacy, and all over the world chair-bound bureaucrats are earnestly compiling toxicological analogues of Mrs. Beeton's cookery book—but without her flair and expertise. How great is the temptation to compile such recipes, seeking to make life easier for both industry and bureaucracy. Every experienced toxicologist realizes the fallacies and pitfalls of such an approach. So too, to their credit, do the Canadian authorities. The accounts presented in this book are intended to be no more than guides that will ensure a high standard of performance along appropriate lines. As such, they constitute a most welcome and valuable addition to the available publications in the areas of carcinogenicity, mutagenicity and teratogenicity.

BOOKS RECEIVED FOR REVIEW

Obesity and its Management. 2nd ed. By D. Craddock. Churchill Livingstone, Edinburgh, 1973. pp. x + 205. £2.50.

Protein Deficiency and Pesticide Toxicity. By E. M. Boyd. Charles C. Thomas, Springfield, 1972. pp. x + 468. $29.50.

Selective Toxicity. The Physico-chemical Basis of Therapy. 5th ed. By A. Albert. Chapman and Hall, London, 1973. pp. xv + 597. £7.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 45. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. vii + 186. DM 43.80.

Practical Histochemistry. By J. Chayen, L. Bitensky and R. G. Butcher. John Wiley & Sons, London, 1973. pp. xiii + 271. £3.95.

Fd Cosmet. Toxicol. Vol. 11, pp. 1121–1138. Pergamon Press 1973. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

AN ASSESSMENT OF THE DELANEY CLAUSE AFTER 15 YEARS*

by

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It is small wonder that the Delaney clause, inserted into the US food-additives legislation as a bulwark against the addition of potential carcinogens to foods, has come to be regarded by many toxicologists as scientifically unsound. During the gestation period of the Food Additives Amendment, nearly 20 years ago, there were even administrators in the regulatory agency who regarded this provision of the law as redundant and it was prophesied that, in time, experience would show it to be impractical. In the past few years, various prestigious groups of scientists have recommended modification of the Delaney clause to permit some latitude for the exercise of scientific judgement in the implementation of its intent. Now, 15 years since enactment of the clause, it is revealing to take a retrospective look at how the 'concept' has been applied in the regulation of pesticide residues and colour and food additives.

Acute and chronic toxicity studies on Aramite®, a highly effective acaricide, were undertaken in 1949, long before the passage of the Pesticides and Food Additives Amendments. The dosage range was set before any information was available on residue levels on fruits and vegetables, and the upper limit of 5000 ppm was believed to be reasonable in view of the low acute oral toxicity (LD_{50} 3·9 g/kg). At the maximum and intermediate doses, hepatic tumours were observed. The study was questioned by the FDA mainly on the ground that it failed to demonstrate a no-effect level. The issue hinged on whether the liver lesion found in only one rat in the group given the lowest dietary level (500 ppm) was carcinomatous or simply hyperplastic. An *ad hoc* advisory committee appointed by the FDA under the terms of the newly enacted Miller Amendment confirmed the opinion of the petitioner's pathologists, and the tolerance (1 ppm) remained in effect. Subsequently, much more extensive carcinogenic studies corroborated the earlier findings in rats (Oser & Oser, *Toxic. appl. Pharmac.* 1962, **4**, 70; Popper *et al. Cancer, N.Y.* 1960, **13**, 1035). However, dogs dosed at 1580 ppm showed marked evidence of hepatic carcinoma (Sternberg *et al. ibid* 1960, **13**, 780) and Aramite was dropped from use on food crops.

The herbicide, 3-amino-1,2,4-triazole, a thyrocarcinogen, was permitted for post-harvest

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use on cranberry bogs provided it left no residue on the next crop. As a result of misuse, residues were found in two lots of cranberries just prior to Thanksgiving Day, 1959. Although more than 95% of all the lots tested were negative, the sale of cranberries came to a virtual halt since it was not possible to trace the distribution of the contaminated cranberries throughout the country.

More recently several of the chlorinated organic pesticides have been banned or threatened on the ground of alleged carcinogenicity. The question concerning dieldrin and aldrin has not been finally resolved despite exhaustive study by NAS-NRC Advisory Committees, while DDT, one of the most effective and economically important pesticides, is to be prohibited by the Environmental Protection Agency, the Administrator having reversed a decision reached by an Examiner after 7 months of hearings that the evidence had not established DDT as carcinogenic.

Among the food additives that have been outlawed under the Delaney concept are natural oil of sassafras and its component, safrole, and oil of calamus, characteristic flavouring components of root beer and vermouth, respectively. Coumarin and its natural source, the tonka bean, were withdrawn from use (principally in artificial vanilla flavourings) when a routine feeding test of a commercial flavour mixture revealed it to be the component responsible for hepatomas in rats.

Approval for the oil-soluble food colourings, FD & C Yellow Nos. 3 and 4, was withdrawn in 1959 following toxicity tests in rats and the demonstration of traces of the bladder carcinogen, 2-naphthylamine. Currently tests are under way on several other certifiable colourings whose non-carcinogenicity has not been 'proven' to the satisfaction of certain critics here and abroad.

Perhaps the most notorious application of the Delaney clause in terms of its effect on the food industry was the summary prohibition of the use of cyclamates in 1969. This followed rather abruptly upon the demonstration of bladder carcinoma in a small proportion of a group of 70 rats fed a diet containing 5% of a 10:1 sodium cyclamate/sodium saccharin mixture (equivalent to 2500 mg/kg). Most of these lesions were evident only microscopically after the animals were sacrificed at 2 years (Price *et al. Science, N.Y.* 1970, **167,** 1131). The study was originally designed to determine whether the effect of this mixture of non-nutritive sweeteners was toxicologically synergistic, the dosage range having been determined on the basis of earlier studies on the individual substances. Nevertheless, the cyclamate alone, rather than the mixture, was assumed by the Department of Health, Education and Welfare to be the carcinogen, a conclusion that may prove to have been unwarranted in the light of recent findings. At the time, however, it seemed justified on the grounds that saccharin had been in use much longer than cyclamate without apparent adverse effects, and some humans were known to convert a small proportion of cyclamate to cyclohexylamine, a compound whose chronic oral toxicity had not been investigated.

That the almost world-wide banning of cyclamates may have been premature is now suggested both by failure to induce bladder cancer in rats given cyclohexylamine at an assumed 10% conversion rate (Morgareidge *et al. Toxic. appl. Pharmac.* 1972, **21,** 330) and by the rumoured finding of bladder tumours in rats fed diets containing 7·5% saccharin. Moreover, studies of cyclamate, saccharin and mixtures of the two in several other laboratories appear not to have confirmed the earlier findings with the mixture.

The basic fault of the Delaney clause lies in the assumption that the ingestion of any substance by animals, irrespective of the magnitude, frequency or duration of dosage, is an appropriate method for determining its carcinogenic potential for man. This concept

flies in the face of fundamental pharmacological principles, not to mention common experience.

There are several separate and distinct parts to the Delaney clause, which states (Code of Federal Regulations, Sec. 409c, 3, A) that a substance is deemed unsafe and hence is proscribed as a direct or indirect component of food. . .

> ". . . if it is found to induce cancer when ingested by man or animal, or if it is found, after tests which are appropriate for the evaluation of the safety of food additives, to induce cancer in man or animal . . .".

Under the first phrase, any substance that is found to induce cancer when fed in any dose to any animal daily for its entire lifetime must be banned from use in food. As administratively construed, this is a legal mandate which leaves no room for scientific judgement as to whether the oral dose is a reasonable one. The phrase following the conjunction "or" made provision for the later adoption of acceptable procedures employing non-oral routes of administration.

The Delaney clause violates the fundamental principle enunciated by Paracelsus in the maxim "Poison is in everything and no thing is without poison. The dosage makes it either a poison or a remedy." The cardinal significance of the dose was recognized by Congress when it substituted the Food Additives Amendment of 1958 for the 'poison *per se* doctrine' implicit in Section 406(a) of the Food, Drug, and Cosmetic Act, and thus provided for the safe use of substances which in higher doses could be toxic.

Pharmacologists and toxicologists have long considered the dose-response relationship to be applicable generally to all chemicals and drugs. Numerous carcinogenesis studies, employing as criteria either the duration of the induction period or the incidence, size and severity of experimentally induced tumours, have demonstrated threshold doses below which no evidence of tumours is found and above which the indicia increase. Oncologists, however, deny the existence of threshold levels of carcinogens. For example, Druckrey (in *Carcinogenesis—Mechanisms of Action*, edited by G. E. W. Wolstenholme and Maeve O'Connor, p. 110, Little, Brown & Co., Boston, 1958) has explained that "The reason why extremely small doses produce no cancer is not that there is a threshold dose, but because the necessary induction time becomes longer than the total life span". Similarly, Weisburger & Weisburger (*Fd Cosmet. Toxicol.* 1968, **6**, 235) have stated that "there are doses for which no tumours are seen over the average lifespan. Were the animals to live longer, tumours could be *predicted* to occur" (italics supplied). This very criterion, however, could be regarded as defining the conditions for establishing a safe dose of a potential carcinogen.

It has been postulated that a single molecule of a chemical may cause a mutation in a single cell sufficient to initiate a malignant process. The hypothesis that sub-threshold doses of a substance may induce unrecognized (and possibly unrecognizable) cellular alterations of a precancerous nature does not justify a conclusion that the substance has been "found . . . to induce cancer" as specified in the Delaney clause. To deny this and contend that "no one knows how to establish a safe dose for a carcinogen" is scientific agnosticism of the first order.

From time immemorial, animal species have throughout life been exposed to low levels of carcinogenic substances introduced into their food or environment either by nature or by man. The variety and multiplicity of these substances would seem to suggest that man, as a species, is capable of surviving such exposures. Some substances known to be carcinogenic when administered orally occur ubiquitously in natural or conventionally prepared

foods. Among them are polycyclic aromatic hydrocarbons in smoked or roasted meats, fish and nuts, mycotoxins in grains, seeds and nuts, oestrogens in soya beans, grains and certain fruits, nitrosamines in cured meats, safrole, asarone and related substances in spices and flavourings, selenium in grains grown on seleniferous soil and ergot in rye flour (Miller, in *Toxicants Occurring Naturally in Foods*, Food Protection Committee, 2nd ed., NAS-NRC, Washington, in preparation 1973). In addition, many substances are known to induce tumours when inhaled or injected subcutaneously, but these routes are not considered appropriate for carcinogenicity testing of food components. The fact that potential carcinogens in common foods are not aetiologically correlated with the incidence of human cancer, supports the probability that trace amounts can be safely tolerated or that the risk, if any, is extremely remote.

Apart from cases of cancer induced by occupational exposure to certain chemicals, evidence correlating the incidence of human cancer with the ingestion of substances demonstrated to be carcinogenic in animals is sparse indeed. It is misleading and fallacious to stigmatize as a 'carcinogen' for man any substance that, under exaggerated conditions, can cause cancer in some species of test animal. In view of the many variables, including species, dosage and route, associated with the experimental induction of carcinogenicity, the term 'carcinogen' should be explicitly defined in the context in which it is used.

For carcinogenesis studies designed to investigate the aetiology or mechanism of the cancer process, the approach is quite different from that appropriate for evaluating the safety of trace substances in the human diet. In the former type of study, highly exaggerated conditions and a variety of dosage routes may be used to shorten the induction time or increase the incidence of neoplasms.

Toxicological tests of food components are based on the premise that for every substance there are toxic and "no-adverse-effect" dose levels, the goal being to determine these levels and evaluate responses in relation to intended or potential uses. A considerable volume of evidence has accumulated to show that carcinogens are no exception to the dose–response relationship. In long-term toxicity studies of food additives, the usual dosage range encompasses a reasonable multiple of the potential human intake and extends high enough to elicit a toxic response. In carcinogenicity testing, however, the highest dose is set at the maximum tolerable level, one that will not materially reduce longevity (Food Protection Committee, "Evaluating the Safety of Food Chemicals", NAS-NRC, Washington, 1970; Joint FAO/WHO Expert Committee on Food Additives—Fifth Report, *Tech. Rep. Ser. Wld Hlth Org.* 1961, **220**; Ministry of Health, "Carcinogenic Risks in Food Additives and Pesticides", Mon. Bull. Minist. Hlth 1960, **19,** 108; Zwickey & Davis, in *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics*, The Association of Food and Drug Officials of the United States, Austin, Texas, 1959). It is interesting that when the total 'lifetime' (2-year) intake of an additive by a 400-g rat is compared with the equivalent intake by a 70-kg man given the same dietary level over a 50-year period, the man:rat ratio is approximately 900.

It may be useful at this point to review briefly the toxicological basis for estimating safe or acceptable levels of food additives and to show that they are not absolute or unequivocal determinations but are subject to many arbitrary experimental and judicious decisions. Some of the pitfalls, which in carcinogenicity testing may lead to false-positive or false-negative results have been summarized by Druckrey (in *Potential Carcinogenic Hazards from Drugs, Evaluation of Risks*, edited by R. Truhaut, p. 60, Springer-Verlag, Berlin, 1967). Among the former are spontaneous tumours, contamination of the test substance, food or

environment, dietary deficiencies, infection with tumour-producing parasites, and various unspecific factors such as the presence of co-carcinogens, hormonal disturbances and the prolongation of life under the test conditions. False-negative results, on the other hand, may result from inadequate actual or effective dosage, insufficient length of study, insensibility of target tissues or the use of a resistant species. Furthermore, chemical and enzymic modifications within the gut may in a given species modify, reduce or enhance the biological effect of a test substance. It has even been shown that responses to intragastric intubation may differ from those produced when the test material is incorporated into the diet (Weil *et al. Toxic. appl. Pharmac.* 1972, **21,** 390).

Toxicologists generally have adopted the position that the no-adverse-effect dose should be based on the observed negative response of a test group, not on extrapolation from the response of higher dosage groups. The uncertainties involved in arriving at a truly maximum no-adverse-effect dose have been discussed previously (Oser, *Fd Cosmet. Toxicol.* 1969, **7,** 415). Particularly at low levels of dosage, the crucial evidence of "adverse effect" is often left to discovery by pathologists. (One could hope for better agreement as to the significance of borderline histopathological aberrations.)

The no-adverse-effect dose, expressed in relation to body weight, is converted to an acceptable daily intake for man by applying a safety factor to compensate for uncertainty in the interspecies transition from test animals to man, for differences in intraspecies susceptibility or resistance and for variations in human dietary patterns. It is interesting to note, however, that in the case of certain essential nutrients, safety factors vary over a wide range (Ostwald & Briggs, in *Toxicants Occurring Naturally in Foods*, Food Protection Committee, NAS-NRC Publ. no. 1354, Washington, 1966). The chronic toxic dose of vitamin A, for example, is only 20–30 times as great as the currently accepted daily allowance, while in the case of sodium chloride the safety factor is only 2. Nevertheless, the factor of 100 has been hallowed by usage and is, in fact, specified by regulations—except when evidence warrants some deviation. A decrease in the safety margin may be justified, for example, if the first effects observed could not occur with lower levels of intake (e.g. osmotic effects) or if they are compensatory rather than pathological (e.g. renal enlargement), if the additive is already present in the diet or body tissues in considerable amount, if the treated food is only rarely consumed or if favourable evidence is available from very long-term human experience. On the other hand, an increase in the safety margin might be indicated for additives proposed for use in staple foods, in foods likely to be eaten in large amounts by children or in foods subject to wide seasonal or other variations in consumption, as well as for additives on which the experimental evidence is incomplete and for those to be used in situations where controls might be inadequate. Moreover, if the effect is transitory or unaccompanied by structural or functional defects, a lower safety factor may be justified than when the effect is severe or irreversible. The slope of the dose-response curve must also be taken into account (Oser, *loc. cit.*).

These, then, are the steps toward estimating acceptable intake levels of substances in food for man. Legal tolerance limits for additives or pesticide residues are set no higher than necessary to achieve their intended functions and must, of course, fall within acceptable dietary ranges.

One of the main difficulties with the Delaney clause, as with other statutory provisions for 'no residue' or 'zero tolerance', has been the continuing improvement, without apparent limit, in the sensitivity of analytical instrumentation and techniques, as a result of which substances prohibited on a 'no residue' basis have later been detected in traces so small as

to be beyond the range of any conceivable toxicological significance (Zweig, in *Essays in Toxicology*, edited by F. R. Blood, Vol. 2, p. 155, Academic Press, New York, 1970). Unless one accepts the dictum of *de minimis non curat toxicologiae*, it becomes necessary in the case of 'carcinogens' to invoke the Delaney clause.

The appraisal of the safety of food additives is a value judgement in which scientific evidence plays only a part, albeit a major one. Not only must the more or less arbitrary nature of the experimental conditions and the subjective aspects of the interpretation of the results be taken into account, but the risks, however remote, must be assessed against the real or potential benefits. In this regard, considerations beyond the scope of toxicology must be weighed. Risk and benefit can be balanced only in terms of socially acceptable judgements.

Because absolute safety is philosophically unattainable, some statisticians insist first on defining some level of permissible risk, say 1:100,000,000 (Mantel & Bryan, *J. natn. Cancer Inst.* 1961, **27**, 455; Weil, *Toxic. appl. Pharmac.* 1972, **21**, 454). But in assessing risk, the toxicologist attaches significance not only to the incidence but to the nature of the observed effect of the substance. Is it transitory or cumulative, functional or organic, mild or severe, and to what extent is it referable to human populations under conditions of use? If the effect is irreversible, as in the case of cancer, is it induced only under extreme experimental conditions, by a dose, for example, exceeding the normal capacity of the animal to excrete or detoxify the substance, and how does this situation compare with the metabolic disposition of the substance when present in the diet of man?

In short, safety evaluation is a multidisciplinary process—not the prerogative of any particular phase of toxicology, be it biochemistry, pathology, oncology or biometrics. It involves not only the judgement of qualified experts, with due recognition of the imprecisions and uncertainties inherent in the evidence, but a realization that the inevitable risk must be sufficiently remote to be socially acceptable.

In conclusion, and for the consideration of those who favour the amendment of the Delaney clause to permit the exercise of scientific judgement in determining the appropriateness of safety evaluation procedures, I would propose the following version (bracketed words omitted, italicized words added):

> ". . . Provided, that no additive shall be deemed to be safe if it is found to induce cancer when ingested by man [or animal], or if it is found, after tests which are *deemed* appropriate *by scientists qualified by training and experience* for the evaluation of the safety of food additives, to induce cancer in man or animal, . . ."

SACCHARIN—A EUROPEAN ASSESSMENT

As a result of the doubts that have been expressed on the safety in use of saccharin, particularly in Japan and the USA, the toxicological data on saccharin were discussed by a group of European Toxicologists. The meeting, the first of its kind, was held at BIBRA, Carshalton, on 12 June 1973. The participants were Professor Gatti (Italy), Dr. Gosselé (Belgium), Professor Lloyd (BIBRA, UK), Professor Magee (UK), Professor Netter (Germany), Professor Peters (Switzerland), Dr. Poulsen (Denmark), Dr. Scott (Eire), Professor Truhaut (France) and Dr. van Esch (Holland), with Dr. Crampton (BIBRA) acting as Chairman.

During the past 3 years a considerable amount of new information on saccharin has been generated by investigations carried out throughout the world This information will

no doubt be published in due course, and it is therefore not appropriate to present the data here. However, the essential points that arise from this work may be summarized as follows:

> Of eight studies which have been carried out using either rats, mice or hamsters and in which various amounts of saccharin have been administered by mouth, malignant bladder tumours have been observed in two studies with rats of the Sprague–Dawley strain. In the remaining six studies, no malignant bladder tumours were observed. However, in one of these, benign bladder papillomas were seen, but as these occurred in both control and test animals these findings were not considered to be relevant to the major question, i.e. is saccharin a carcinogen to animals or man?

In attempting to answer this question, the possible reasons for the discrepancy between the results of different experiments were examined. The two studies in which tumours were observed showed a number of common features. Tumours were seen only at the highest levels of saccharin intake, i.e. at 5 and 7·5% saccharin in the diet. The rats were exposed *in utero* by feeding saccharin to pregnant females and the offspring were subsequently fed for their lifespan. In comparison with control rats, the following effects were observed: a smaller number of litters, fewer live births, an increase in the death rates of neonates, a lower birth rate, a lower incidence of fertile matings and a lower number of implantations *in utero*. In addition, the newborn showed a slower growth rate over their lifespan, although food consumption was not affected. During the first year of life, the animals had lower haemoglobin, haematocrit and transaminase levels than the control animals. The conclusion reached by the meeting was that only in animals that showed the toxic effects of high saccharin intake were tumours observed.

The appearance of bladder tumours only in animals exposed to toxic levels of saccharin *in utero* is difficult to interpret. It is known that changes in immunological competence in early life may influence the appearance of tumours in later life but the extent, if any, to which saccharin at high doses may affect immunological responses could not be assessed from the data.

Data on the chemical specification of saccharin illustrated another possible explanation for differences in experimental results. Saccharin produced by a process using toluene as the starting material is known to contain *o*-toluenesulphonamide as a major contaminant, and recent data have shown that this may be present in amounts up to 5600 ppm. The carcinogenic potential of this compound has never been investigated, though compounds of similar structure are known to produce bladder tumours in rodents. Unfortunately the actual content of *o*-toluenesulphonamide in the saccharin used in the animal studies is not known, and therefore the possibility that the two studies in which bladder tumours occurred involved high levels of this contaminant could not be ascertained. However, in view of the high levels of 5 and 7·5% of saccharin used in these studies, the effect of this contaminant is clearly important.

One factor known to induce bladder tumours is the presence of stones within the bladder. Among the various studies, some observations of stone formation were recorded, but the data were inadequate to assess the extent, frequency and type of stone formation that occurred. However, one study in which saccharin was administered in the drinking-water showed that marked increases in urinary pH occurred, with subsequent formation of urinary concretions, sediment and gritty calculi in male rats. Although the explanation for these effects is not known, it suggests that stone formation might have been a factor in the development of the observed bladder tumours.

Other facets of the studies were discussed; these included susceptibility of rodent strains to bladder carcinogens, the composition of the basic animal diets used in the various studies, metabolism of saccharin, transplacental transport of saccharin, enzyme inhibition by saccharin and its contaminants, and the significance of other histopathological observations.

The extent to which further research could elucidate the factors likely to be responsible for the differing results of these saccharin studies was discussed, and particular note was taken of three human epidemiological studies which are currently in progress. The benefits of saccharin to the normal and diabetic population were also considered.

It was recognized that in the USA there is a legal requirement, owing to the existence of the Delaney clause, to consider urgently any findings that may indicate that a food additive has carcinogenic properties. It was further recognized that decisions made in the USA may have, and in the past have had, repercussions in other countries over and above the scientific data being considered. For these and other reasons, it was deemed desirable for the meeting at BIBRA to express and record its opinion and judgement on the data, independently of other assessments in the USA or elsewhere.

The conclusions reached were as follows:

(1) There was unanimous agreement that the data did not justify the banning of saccharin. The opinion of all participants was that the data did not demonstrate that saccharin had carcinogenic properties. It was also unanimously agreed that the appearance of bladder tumours in two experiments needed explanation. Clearly, the special features of the experimental design of these studies should be considered.

(2) In view of the presence of *o*-toluenesulphonamide in varying and often appreciable amounts in production batches of saccharin, a recommendation was made by a considerable number of participants that consideration be given to placing a limit on the amount of this impurity, pending a specific investigation of its toxicological and carcinogenic potential.

(3) With few exceptions, participants recommended that the subject be reviewed at a time when the results of epidemiological studies became available.

(4) Two of the participants, while recognizing that evidence on the carcinogenicity of saccharin was inconclusive, favoured some limitation of its use in various products. The opinions expressed on this point included a limitation or possible ban on saccharin in baby foods, and perhaps in products largely consumed by young children. Concern was also expressed on the possibility of a high saccharin intake by workers, the nature of whose jobs tended to favour a state of dehydration and hence the formation of concentrated urine. However, no consideration was given to how much a restriction could be brought about in practice.

(5) One opinion was that the ADI for saccharin should be officially recognized as 5 mg/kg, which is the lower of the two recommendations proposed by the Joint FAO/WHO Expert Committee on Food Additives. Such an official recognition would be temporary, depending upon the results of further work as indicated earlier in this report.

(6) Because of the extensive consumption of saccharin by large populations for 80 years with no apparent adverse effects in man, another opinion was that no immediate restriction should be placed on saccharin consumption, on the understanding that industry and/or government agencies would undertake or finance the work deemed necessary to elucidate the problems defined by present data.

Summary—The data do not demonstrate that saccharin is a carcinogen. The results of further experimental and epidemiological studies should be considered before any final conclusion can be made. In the interim, specifications relating to the purity of saccharin should be re-examined, and consideration should be given to the future use of saccharin in special situations such as infants' and children's food.

PNEUMOCONIOSIS

Coal-workers pneumoconiosis (CWP), the respiratory disease that affects miners, has attracted much attention in recent years. Concern for the sufferings of the affected miners and an awareness of the considerable incapacity that results from years of working in the mines, has prompted a great deal of study on the factors involved in the development of CWP. These studies demand a multidisciplinary approach in the widest possible sense, involving not only scientists and clinicians but also social workers and anthropologists. It is not surprising, therefore, that the number of publications on this topic has reached staggering proportions. Moreover, because of the wide range of interests involved, the literature is scattered through a wide variety of journals, so that keeping abreast of developments in this field is a particularly difficult task.

The appearance in a single volume* of an exceptionally comprehensive series of contributions on the various aspects of the CWP problem must therefore be viewed wth enthusiasm. This excellent collection of papers, originally presented at the International Conference on Coal Workers' Pneumoconiosis held by the New York Academy of Sciences in September 1971, provides an overall survey of recent studies on the extent, distribution, diagnosis, treatment and prevention of this disease, and on the pulmonary changes, both functional and pathological, associated with it and the specific materials that cause or exacerbate these changes.

The papers are presented under nine main headings, several of which cover topics of major interest to toxicologists. Considerable attention is paid to the coal-mine dust itself and to methods for its analysis. This occupies the opening section of the proceedings, an appropriate arrangement since it is this dust which is the principal factor in the aetiology of lung disease in coal workers. Respirable coal dust is a mixture of carbonaceous particles, various other organic constituents and mineral deposits such as quartz, silica and trace elements. It has now become possible to measure the amounts of these various constituents in the dust much more accurately than in the past as a result of developments in polarography, spectrometry and the neutron activation technique (Freedman & Sharkey, p. 7). Corn *et al.* (p. 17) analysed samples of "respirable" coal-mine dust from two mines in the Pittsburgh, Pa. seam. A wide range of trace metals was found, and some indication of the mineral content can be gained from the ash figures, which were in the 8·5–10% range. The size of the particles was closely investigated, about half being found to be below 3·4 μm and therefore of a size which enabled them to penetrate readily into the alveoli. The size of a high proportion of particles was found to be 1·7 μm or below.

Coal Workers' Pneumoconiosis.* Edited by I. J. Selikoff, M. M. Key and D. H. K. Lee. *Ann. N.Y. Acad. Sci.* 1972, **200. pp. 861. Page numbers accompanying authors' names in the above review refer to the initial page of the relevant contribution in this publication.

The biological processes available for the disposal of any dust that does penetrate into the alveoli involve macrophage uptake of the particle, followed by transfer of the macrophage across the alveolar epithelium (possibly by passage between the epithelial cells) and its deposition, together with the particle, in the lymphatic tissues. This disposal mechanism is considered in papers by Albert & Lippmann (p. 37), Morrow (p. 46) and Ferin (p. 66). They indicate quite clearly that a fairly efficient system exists for keeping the alveoli (in which gaseous exchange takes place) free from obstruction. However, the dust particles are not susceptible to destruction by the macrophages so that, over the years, substantial deposits build up in the lymphoid tissue around the bronchi, including the lymph nodes in the region of the main bronchi and the trachea.

Pulmonary deposits of inert particulate material, such as bituminous particles with a high carbon content, do not appear to lead irrevocably to pathological conditions that impair health (Lapp & Seaton, p. 433; Naeye, p. 381). However, the changes produced are evidently less trivial than they at first appear, since there is some evidence that they may impair the functional capacity of the lymphatic system. People affected in this way are more susceptible to the development of emphysema (Heppleston, p. 347) and chronic bronchitis (Ryder *et al.* p. 370) and exhibit some impairment of certain immunological responses (Burrell, p. 94). Experimental evidence also supports the possibility that they may be more susceptible to tuberculosis (Gernez-Rieux *et al.* p. 106).

The presence of silica and quartz in coal-mine dust leads to direct injury to the lung. Such dusts are lethal to macrophages (Naeye, p. 381) and induce a chronic granulomatous process which is accompanied by fibrosis and eventually results in the destruction of a substantial part of the lung tissue and a marked reduction in ventilatory capacity. The disability associated with this pathological process (Heppleston, p. 347; Pratt, p. 342; Ryder *et al.* p. 370) renders the victim unfit for work and in its severest form may confine him to a wheel chair. Of course, these developments represent the advanced stages of the disease (Lapp & Seaton, p. 433). In its earlier stages, very little physiological disturbance occurs and the disease may not be recognized unless physiological tests of lung function, including an assessment of gas transfer, are carried out (Rasmussen, p. 455). An alternative way of detecting CWP is by radiological examination of the chest (Heitzman *et al.* p. 510; Rossiter, p. 465). This can be a very sensitive index of the disease, but it is important to remember that it is fraught with pitfalls, since a number of other lung diseases may present a radiological picture very similar to that of CWP (Pendergrass *et al.* p. 494).

Considerable attention has been paid to the epidemiology of CWP, and several important studies carried out in the major coal-producing countries were reported and discussed at some length at the meeting and comprise Part III of the published proceedings. Part VII is concerned with another important aspect of the problem—measures that may help to prevent or retard the development of the disease and the types of treatment that are available.

Perusal of the 62 papers in this volume and of the discussions they provoked gives a clear impression of the vast amount of effort that is being spent in attempts to combat this major industrial disease. While the many facets of this problem are being appraised in relation to the health of the miner, particular stress is being laid, quite rightly, on the identification of those aetiological factors that offer some possibility of being eliminated from the mining environment.

ANOTHER FLUORIDE CHAPTER

Introduction

The hazards of fluoride have been known for years, and its benefits have also been well-publicized in the past decade or two. Its distribution is virtually universal, though uneven, and its absorption from the stomach and intestine depends upon the presence of other dietary ions, notably of calcium (Ca), aluminium (Al) and magnesium, with which it forms relatively insoluble and therefore feebly absorbed complexes (Shupe *et al. Clin. Toxicol.* 1972, **5,** 195). After distribution throughout the plasma and soft tissues, fluoride is selectively deposited in the skeletal and tooth matrices, and some 98% of the body content is found in bones. Foetal blood carries roughly the same concentration of fluoride as the maternal blood, and again selective deposition occurs in foetal bone and developing teeth.

Fluorosis in animals

Chronic fluoride intoxication is most often observed in livestock. It usually develops gradually, producing signs that may easily be mistaken for advancing osteoarthritis or trace-element imbalance. The main characteristic which enables diagnosis to be made is dental fluorosis, involving the mottling of enamel, hypoplasia, leading to defective enamel, and hypocalcification, particularly in the incisor teeth (Shupe *et al. loc. cit.*).

Griffith Jones (*Vet. Rec.* 1972, **90,** 503) has reported that a high incidence of hip arthritis in a dairy herd of Ayrshires was associated with excessive fluoride levels in bone samples. There were no marked dental lesions to reveal the intoxication, since dentition was complete before the herd received the mineral supplement containing 4974–6994 ppm fluoride, which was found to be the source of the excess. The level of fluoride in the total dry feed amounted to 50–70 ppm. In this herd, arthritis was the most prominent feature, and led to advancing debility and loss of milk and a reduction in carcass value.

Gründer (*Zentbl. VetMed. A* 1972, **19,** 229) asserts that air pollution by fluoride from modern industrial processes, especially the extraction of Al, is a serious hazard for plants, animals and man. Fluoride produced by many combustion processes is continually raising the fluoride content of soil, surface-waters and air in inhabited regions of the earth. In cattle, which are particularly susceptible to excess fluoride, uptake results in tooth fluorosis, which may be tolerable, and, at higher levels, in degrees of tooth and bone fluorosis which are frankly toxic. Gründer (*loc. cit.*) describes how the amount of fluoride absorbed by herds of cattle grazed either near a factory producing hydrofluoric acid or near another producing Al was successfully reduced by Al salts added to the food or drinking-water.

In a direct experiment, Sundström (*Acta path. microbiol. scand. A* 1972, **80,** 17) administered 1 or 5 ppm fluoride in the drinking-water, as the sodium salt, to small groups of rats for 2 years. Of the 16 rats given fluoridated water, 13 survived for the whole term of the experiment, and radiologically demonstrable cavities due to resorption of bone were found in the femurs of four of these (three given 1 ppm and one given 5 ppm fluoride). None of the animals developed tooth lesions. It should perhaps be noted that in this study, as in another producing similar results (Röckert, *ibid* 1963, **59,** 32), distilled water was used as the vehicle for the fluoride. Gedalia *et al.* (*Archs int. Pharmacodyn. Thér.* 1960, **129,** 116), who gave rats tap-water containing up to 10 ppm fluoride, found no structural changes in the femurs after 9 months.

Effect on reproductive function

The effect of feeding diet containing 30, 150 or 450 ppm fluoride to pigs, in conjunction

with dietary supplements of 0·5% Ca and 0·4% phosphorus (P) or 1·2% Ca and 1·0% P, has been studied by Forsyth *et al.* (*Nutr. Rep. Int.* 1972, **5,** 313). Growth studies indicated that 150 ppm was close to the maximum acceptable level for finishing pigs and proved to be excessive in piglets (from 4 weeks of age), decreasing feed consumption and impairing growth. The Ca level of the diet did not affect the rate of weight gain or feed efficiency in the presence of fluoride. Reproduction in first-generation females was not significantly altered by 150 ppm fluoride but conception difficulties in all control and test groups (0, 30 and 150 ppm fluoride) made the results inconclusive. On the other hand, results reported by Devoto *et al.* (*Archs oral Biol.* 1972, **17,** 371) indicated that excess fluoride had toxic effects on the rat placenta and that these sometimes led to foetal death. Pregnant rats were given sodium fluoride in ip or sc doses of 1, 5, 10, 15 or 20 mg/kg body weight daily from day 10 to day 18 of gestation. Foetal deaths *in utero* were more frequent in all the treated groups than in the controls, and their incidence was paralleled by the number of necrotic placentae. The effect was significantly greater with ip than with sc injections, indicating higher exposure of the placenta when injection was by that route. The dose-response relationship was weak, possibly on account of damage to the peritoneum and subsequent alteration of the rate of fluoride absorption. The experiments strongly suggested that the toxic action of fluoride occurred at the placenta, and that foetal death was secondary to this.

Effect on the ear

Schätzle & von Westernhagen (*Arch. klin. exp. Ohr.-, Nas.- u. Kehlk Heilk.* 1971, **200,** 292) have reported an unusual effect of fluoride on the organ of Corti in guinea-pigs. After ip injections of 0·1 mg sodium fluoride twice daily for 10 days, little morphological change could be detected in the cochlea, but the organ of Corti was found to be deficient in a number of enzymes. The most important of these was acid phosphatase, which was also depleted in the labyrinth of the treated animals. This aspect is obviously one to be borne in mind in connexion with the suggested use of fluoride in the treatment of otosclerotic disease or bone tumours.

Biochemical effects

Other effects on enzyme systems were described in a paper by Shearer & Suttie (*J. Nutr.* 1970, **100,** 749), who found that a single ip injection of 2 mg fluoride to rats substantially increased the fluoride concentration in the liver and brought about changes in metabolite levels apparently related to the inhibition of enolase. Dietary administration of 450 or 600 ppm for 3 days decreased most glycolytic intermediates, and in particular pyruvate and lactate, and greatly increased liver citrate. Fluoride ingestion also decreased the activity of liver pyruvic kinase but had no apparent effect on enolase. Apart from the rise in citrate production, however, the changes in metabolism appeared to be secondary to the altered amount and pattern of food intake induced by the addition of fluoride to the diet. Shearer *et al.* (*ibid.* 1971, **101,** 1037) found in an extension of this work that when rats were fed a diet containing 450 or 600 ppm fluoride, the liver and blood citrate levels rose after 3 days but returned to normal within 2 weeks, while the citrate content of the femurs, which had fallen after 3 days, still remained depressed at the end of 2 weeks. Continuous iv infusion of 3 or 10 mg citrate daily for 3 days failed to increase the citrate level in blood or liver, however, and bone slices from the rats fed fluoride for 3 days showed no alteration from controls in their capacity to incorporate [2-^{14}C]acetate into citrate or total organic acids. Neither were the oxidation of citrate to CO_2 and the citrate concentration in kidney affected.

When 20 ppm fluoride was added to isolated perfused rat liver, the citrate levels did not change although the concentration of total glycolytic intermediates did. Feeding the fluoride-containing diets for 3 or 14 days had no observable effect on the activity of liver aconitase, isocitrate dehydrogenase, malate dehydrogenase, citrate-cleavage enzyme or citrate-condensing enzyme. Thus the metabolic effect of fluoride leading to elevation of blood and liver citrate in rats remains unidentified. Evidently it is not simply a question of fluoride-induced release of bone citrate, inhibition of citrate oxidation, or inhibition of citrate-metabolizing enzymes in the liver.

On the other hand, the effect of fluoride on lipid metabolism in bone seems clear-cut, according to a paper by Wolinsky & Guggenheim (*Israel J. med. Sci.* 1971, **7**, 527), who supplemented the drinking-water of rats with 200 ppm fluoride for 2 weeks and afterwards isolated lipids from the metaphyseal and diaphyseal bones of femurs and tibias. The fluoride treatment not only caused a significant reduction in body-weight gain and the citrate content of bone but significantly decreased the bone level of lipid (triglyceride plus phospholipid). At the same time the incorporation of [2-^{14}C]acetate into the lipid fractions was markedly reduced, from 86 to 59%.

Effects in man

Ferguson (*Nature New Biology* 1971, **231**, 159) studied the effects of controlled daily doses of 5 mg sodium fluoride for 3 months on volunteers. This dose was roughly equivalent to drinking water containing 5 ppm fluoride. Serum alkaline phosphatase (AlkPase) activity was reduced by about 20% during the first 6 weeks, but serum-protein patterns were not significantly affected. A further survey of serum AlkPase in a group of 50 students given drinking-water containing 1 ppm fluoride for 3 months showed that the enzyme level fell by 16% after 4 weeks but had returned to pre-test levels by week 8. In another group given fluoridated water with 0·43 ppm fluoride for 4 weeks and thereafter with 0·9 ppm, serum AlkPase decreased initially, returned to base level, decreased again when the fluoridation level was doubled, and then reverted once more to base level in a further 6 weeks. The findings in these studies and subsidiary tests indicate that this reduction in serum AlkPase activity cannot be explained by any direct inhibition of the enzyme by fluoride, and the observed adaptation to increased fluoride intake supports the view that protein changes take place in the affected enzyme. Lowered serum AlkPase might result from a reduction in the liberation of enzyme from tissue stores, but such a presumed change in cell-membrane permeability would not explain the phenomenon of adaptation. A more likely explanation of the effect of fluoride is considered to be the induction of an alternative pathway of AlkPase production in the tissues.

A recent Editorial (*British Medical Journal* 1972, **4**, 748) has drawn attention to reports of osteoporosis surveys carried out in areas of high and low fluoride concentration in North Dakota and indicating that high-fluoride areas may have some protective influence on the loss of bone substance in this disease, but the Editorial also stressed the existence of contradictory findings. A criticism of the suggestion by Jowsey *et al.* (*Am. J. Med.* 1972, **53**, 43) that fluoride therapy may be effective against severe osteoporosis has been offered by Dequeker & Burssens (*Br. med. J.* 1973, **1**, 551), who maintain that the limits of experimental error inherent in the reported observations preclude the drawing of firm conclusions and stress that the large-scale treatment of osteoporotic patients with sodium fluoride must await further evidence of its efficacy and safety. Ryckwaert *et al.* (*Revue Rhum. Mal. ostéo-artic.* 1972, **39**, 627) have reported the results of giving sodium fluoride at a level of 1–1·5 mg/

kg/day for 12–31 months to 14 patients with primary osteoporosis. In half the group there was an increase in the volume of bone, while the rest showed either an insignificant increase or a decrease. In ten of the 11 patients treated for more than 2 years the volume of osteoid tissue was found to have increased, while the proportion of bone surface covered with osteoid at calcification fronts had diminished. The fluoride certainly disturbed the mineralization process in osteoporotic bone, but bone resorption increased in most patients. Thus, this study provided little if any support for the clinical treatment of osteoporosis with fluoride.

Not only does fluoride adversely affect the mineralization of bone (*Cited in F.C.T.* 1971, **9,** 278); a high intake may also aggravate symptoms of arthritis. Cook (*Lancet* 1971, **ii,** 817) related the case of an arthritic woman who was a heavy tea-drinker and as a result consumed more than 9 mg fluoride daily. She was persuaded to stop drinking tea, whereupon her daily fluoride intake dropped to below 1 mg. During the following year she experienced a considerable reduction in pain and became much more mobile, an improvement which was subsequently maintained although further amelioration of the condition did not occur. On the strength of this case, Cook (*loc. cit.*) suggested that subclinical fluorosis, which was not demonstrable radiologically, might sometimes be responsible for pain diagnosed as rheumatic or arthritic.

One problem raised by fluoridation lies in the inability of some individuals to excrete fluoride adequately on account of kidney dysfunction. Juncos & Donadio (*J. Am. med. Ass.* 1972, **222,** 783) have described two young patients with renal impairment associated with polydipsia and polyuria. When they consumed large volumes of highly fluorinated natural water (containing 2·6 and 1·7 ppm fluoride, respectively) both developed systemic fluorosis which was clinically and radiologically evident.

Finally, as an example of what happens when very large quantities of fluoride are ingested, there is the case reported by Abukurah *et al.* (*ibid* 1972, **222,** 816). A young man swallowed about 120 g of a roach bait, 97% of which consisted of sodium fluoride, in a suicide attempt. He suffered from nausea and vomiting and was taken to hospital where his stomach was washed out with lime water. He went into tetany despite calcium gluconate injections, and this was followed by respiratory arrest and ventricular fibrillation. Many applications of direct-current countershock were necessary to maintain his heart beat, and eventually a cardiac pacemaker was inserted as a temporary measure. Intensive treatment had to be carried out for 12 hours before the arrhythmias ceased. An oesophageal stricture later developed and the patient had to be fed through a gastrostomy for 27 days. As the authors of the paper comment, recovery from such a heavy dose of fluoride as this was unexpected and was only possible because of the intensive treatment facilities immediately available.

POTATOES AND NEURAL TUBE DEFECTS

Some recent reports have suggested a possible association between the consumption of blighted potatoes and a relatively high incidence of two congenital malformations, spina bifida and anencephaly, which for convenience are sometimes designated ASB although they do not form a joint entity. Since our previous review on this subject (*Cited in F.C.T.* 1973, **11,** 311), several additional contributions have appeared in the medical literature,

and on the whole these seem to present a rather different picture, although the question cannot be considered to be by any means settled.

The author of the original hypothesis has made further contributions to the correspondence (Renwick, *Lancet* 1973, **i,** 96; *idem*, *Br. med. J.* 1973, **1,** 172), replying to his critics and enlarging on his earlier points, but on the other side of the fence there have now assembled reports of several additional epidemiological studies. Thus Spiers (*Lancet* 1973, **i,** 426) has compared the incidence of ASB in the north-eastern and south-eastern States of the USA, noting that potato loss due to blight appears to be markedly higher in the former than in the latter region. He found no relevant difference in the ASB incidence in these areas; in fact he found that in the decade 1958–1968, death rates from spina bifida among white infants were higher in the south-east. The situation is somewhat complicated, however, by the apparent shipment of large quantities of potatoes from northern to southern areas.

Nearer home, Smith *et al.* (*ibid* 1973, **i,** 269) analysed ASB data for the Edinburgh area for the 17-year period 1954–1971 and were able to find no correlation between the annual fluctuations in the incidence of these congenital defects and the severity of potato blight during the year in which the affected infants were conceived. Similarly, a survey carried out by MacMahon *et al.* (*ibid* 1973, **i,** 598) on the incidence of neural-tube defects in Boston between 1930 and 1964 revealed no correlation between the incidence of ASB and either the annual severity of potato blight in Maine, which supplies the bulk of the potatoes eaten in Boston, or seasonal variations in the consumption of blighted potatoes.

Another investigation was carried out by Elwood (*ibid* 1973, **i,** 769) in the eastern provinces of Canada (Nova Scotia, Prince Edward Island, New Brunswick and Quebec), where late potato blight occurs with periodic severity and where potato consumption is about 80% of the British consumption. The incidence of infant deaths and stillbirths from anencephaly was calculated from data supplied by Statistics Canada and was compared with estimates of potato-blight severity in plants and tubers taken from the Canadian Annual Plant Disease Reports. No evidence was found that the annual variations in mortality from anencephaly in these provinces were related to changes in blight severity. Further support for the view that potato blight was unlikely to be causally related to anencephaly was provided by a parallel study in Ontario during the same 24-year period (1944–1968). This province has considerable potato production but little blight and yet the anencephaly figures did not differ markedly from those of the other provinces (Elwood, *loc. cit.*).

Moving from epidemiology to experiment, raw or boiled homogenate of blighted potatoes failed to induce any foetal abnormalities when administered to pregnant rats by gastric intubation from day 5 to day 11 of pregnancy (Chaube *et al. ibid* 1973, **i,** 329). This result supported the findings in an earlier study in rats (*Cited in F.C.T.* 1973, **11,** 313).

These failures by several workers to correlate potato-blight severity with an increase in the incidence of ASB are worthy of note. Equally interesting are the comments by Carter (*Br. med. J.* 1973, **1,** 290), Baird (*ibid* 1973, **1,** 291) and Cruz-Coke (*Lancet* 1973, **i,** 269), who have questioned the validity of the earlier conclusions drawn on the basis of data compiled from the UK and Chile. For what it is worth, the existing experimental evidence does not seem to provide much support for the original hypothesis either.

BREATHING SULPHUR DIOXIDE

Introduction

Sulphur dioxide (SO_2) is one of the commonest pollutants of the atmosphere in inhabited regions. Urban concentrations are usually in the region of 0·2 ppm, but local maxima may reach 2 ppm (*Cited in F.C.T.* 1970, **8,** 219). Since airway resistance has been increased in some sensitive individuals by 5 ppm SO_2 adsorbed on particulate matter, there are no grounds for complacency, however laudable the recent improvements in urban atmospheres. At 12–15 ppm, SO_2 causes irritation of the nasal mucosa in animals and suppresses the ciliary activity which normally clears particles from the upper respiratory tract (*ibid* 1970, **8,** 219).

Local effects on the respiratory tract

The exposure of mice to 10 ppm SO_2 has produced more severe tissue injury in the nasal cavity than in the trachea or lungs (Giddens & Fairchild, *Archs envir. Hlth* 1972, **25,** 166), particularly in those animals which showed prior evidence of mild infection of the upper respiratory tract. This correlates with the physiological evidence indicating that most inhaled SO_2 is absorbed through the nasal mucosa during normal nasal breathing. The nasomaxillary turbinates of the mice in this experiment developed lesions, which included oedema, necrosis and desquamation of the respiratory and olfactory epithelium, after 24-hour exposure to 10 ppm SO_2. In another experiment, specific-pathogen-free piglets were continuously exposed to about 35 ppm SO_2 for 1–6 weeks (Martin & Willoughby, *J. Am. vet. med. Ass.* 1971, **159,** 1518) and developed varying degrees of ocular and nasal irritation, with motor depression. After 1–2 days, the severity of the irritation diminished. A rise to nearly 100% in the relative humidity of the air which these pigs breathed enhanced the irritation produced by SO_2, however; a sobering thought for those who dwell in humid climates. Histopathological changes affected the epithelium of the nose and trachea, with ciliary loss, reduction in the number of goblet cells, enlargement of the nuclei of the epithelial cells, disappearance of numbers of mature cells, and metaplasia. The lungs of these animals showed no changes.

In another study by the same two authors (*idem*, *Archs envir. Hlth* 1972, **25,** 158), piglets were continuously exposed for 1–6 weeks to corn dust, or to corn dust or corn starch in association with 33–36 ppm SO_2. Corn dust alone provoked no observable reactions, although histological examination showed slight thickening of the epithelium of the trachea and bronchi and an increase in the numbers of goblet cells in the trachea. When SO_2 was blown into the exposure chamber with either of the dusts, ocular and/or nasal irritation occurred, with salivation and depression. After 2 weeks, the cilia of the trachea were shortened and the number of goblet cells was decreased. This effect progressed, until at week 6 substantial thickening of the tracheal epithelium and shortening of the cilia were seen, although the numbers of goblet cells appeared normal again. More striking damage was evident in the epithelium of the turbinates.

Goldring *et al.* (*ibid* 1970, **21,** 32) exposed hamsters with experimental papain-induced emphysema to an average of 650 ppm SO_2 for 4 hours daily on 5 days each week for a total of 19–74 days. At this concentration, SO_2 provoked lachrymation, coughing, nose-scratching and excessive activity at first, but within a few days tolerance developed, although the exposed animals continued to weigh somewhat less than the controls. By the end of the experiment there was a significant incidence of mild bronchitis, but this was not associated

with any marked alteration in the mechanical properties of the lung. In other words, the SO_2 exposure did not aggravate the emphysema. In hamsters exposed to SO_2 without prior induction of emphysema, mild inflammatory changes in the bronchial tree were observed. Thus, SO_2 in these experiments failed to contribute significantly to the development of obstructive lung disease.

Effect on pulmonary function and infection

In a series of experiments reported by Alarie *et al.* (*ibid* 1970, **21,** 769), three groups of guinea-pigs were exposed continuously for 12 months to 0·13, 1·01 or 5·72 ppm SO_2. Animals killed after exposure to 5·72 ppm SO_2 showed a reduction in the incidence and severity of spontaneous pulmonary disease, with a lack of histiocyte infiltration into the alveolar walls. The controls and those exposed to the lower concentrations of SO_2 showed tracheitis. After 52 weeks, animals with the highest exposure showed a definite reduction in spontaneous pulmonary disease, and no detrimental effects on pulmonary function. An incidental finding was a greatly reduced incidence of nephritis and nephrosis in guinea-pigs exposed to 5·72 ppm SO_2, but these same animals showed a marked degree of cytoplasmic vacuolation in the liver accompanied by an increase in the size of the hepatocytes.

Further experiments by Alarie *et al.* (*ibid* 1972, **24,** 115), this time in cynomolgus monkeys, demonstrated a lack of ill effects on the lungs of monkeys exposed to 0·4 or 1·28 ppm SO_2 for 24 hours daily, 7 days/week for 78 weeks. No alteration in pulmonary function and no histological damage to pulmonary tissues could be demonstrated in these animals. On the other hand, in a group of monkeys continuously exposed to 4·69 ppm SO_2 for 30 weeks, followed by accidental over-exposure to between 200 and 1000 ppm for 1 hour, microscopic alterations were apparent in the bronchioles, alveolar ducts and alveolar sacs, with moderate bronchiectasis and bronchiolectasis but little evidence of fibrosis. In the over-exposed group, deterioration of pulmonary function persisted throughout the subsequent 48-week observation period.

Studies of pulmonary function in anaesthetized cats exposed, by means of mechanical ventilation, to SO_2 with and without sodium chloride (NaCl) aerosols have been reported by Corn *et al.* (*ibid* 1972, **24,** 248). No significant increase in pulmonary flow resistance occurred until the concentration of SO_2 reached about 20 ppm, and most animals failed to show any significant pulmonary response even to this concentration of SO_2, with or without NaCl aerosol (10 mg/m^3). Changes in flow resistance became more frequent when SO_2 was delivered by endotracheal catheter or face mask. When exposure to SO_2 was interrupted, normal pulmonary function was rapidly restored.

We have seen that 1 ppm SO_2 in conjunction with carbon particles failed to change the bacterial flora of the respiratory tract in rats (*Cited in F.C.T.* 1970, **8,** 219). Experiments by Zarkower (*Archs envir. Hlth* 1972, **25,** 45) have shown that in mice exposed to 2 ppm SO_2, to particulate carbon or to both for 192 days, there was a decrease in antibody-forming response to challenge with *Escherichia coli* antigen. However, after 102 and 135 days of exposure to SO_2, antibody production in the mediastinal lymph nodes of the mice was enhanced, only to be reversed later. Antibody enhancement was particularly marked in the spleen at 135 days, and was greatest in mice exposed to both carbon and SO_2. Some such effect may explain the results obtained by Alarie *et al.* (1970, *loc. cit.*) in guinea-pigs. On the other side of the picture, however, is the finding of Fairchild *et al.* (*Archs envir. Hlth* 1972, **25,** 174) that mice infected with influenza and then exposed to 20 ppm SO_2 continuously for 7 days developed more pneumonia than did control animals with influenza.

The threshold for an increase in the incidence of post-influenzal pneumonia was found to be about 7–10 ppm SO_2. Moreover, prior exposure to 25 ppm SO_2 rendered mice more susceptible to pneumonia following an influenza inoculation. Exposure to SO_2 did not affect the growth of influenza virus in the lungs, but the mild inflammatory lesions induced by SO_2 apparently favoured the development of pneumonia, the severity of which depended on the SO_2 concentration to which the animal was exposed. Fairchild *et al.* (*loc. cit.*) confirmed, in the low range of 2·9–4·3 ppm SO_2, the lowered liability to lung infection noted by Alarie *et al.* (1970, *loc. cit.*) with a concentration of 5·72 ppm.

Other effects

Bloch *et al.* (*Archs envir. Hlth* 1972, **24,** 342) have described the development of mitral stenosis in one dog exposed for 5 years to raw automobile exhaust plus oxides of sulphur (0·14 mg/m^3) and of mild systemic arterial hypertension in another exposed to oxides of sulphur alone. The significance of these findings is not clear. Another isolated observation comes from P'an & Jegier (*ibid* 1970, **21,** 498), who found that *in vitro* 4 ppm SO_2 or more inhibited bovine erythrocyte acetylcholinesterase (AChE) activity. Concentrations lower than 4 ppm had only a slight and erratic effect on AChE. The effect of SO_2 was, however, more pronounced than that of an equivalent concentration of $NaHSO_3$. From the graphic relationship between the SO_2 concentration in the preparation and the relative AChE activity, P'an & Jegier (*loc. cit.*) derived the formula: $y = 100 - be^x$, where y is the relative AChE activity and x is the pollutant concentration. The value of b was found to be 2·5 for SO_2 and 4·3 for ozone.

Fd Cosmet. Toxicol. Vol. 11, pp. 1139–1164. Pergamon Press 1973. Printed in Great Britain

TOXICOLOGY: ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

2611. More about allyl alcohol and acrolein

Reid, W. D. (1972). Mechanism of allyl alcohol-induced hepatic necrosis. *Experientia* **28,** 1058.

Allyl alcohol (AA) was shown to cause extensive periportal necrosis of the rat liver more than half a century ago. It has been demonstrated *in vitro* (*Cited in F.C.T.* 1973, **11,** 325) that AA can be converted by alcohol dehydrogenase, found mainly in the periportal area of the liver, to acrolein, which—like AA—inhibits mitochondrial respiration and blocks protein synthesis. The present study yields the first direct *in vivo* evidence that AA produces periportal necrosis through the binding of a metabolite to periportal hepatocytes.

Rats pretreated with either saline or pyrazole (an inhibitor of hepatic alcohol dehydrogenase) were given [^{14}C]AA (0·05 ml/kg, ip) after an interval of 2 hr and were killed 8 or 24 hr later. Another group of animals was pretreated with phenobarbitone, to induce microsomal enzymes, before AA treatment. An autoradiographic study was carried out on liver, lung and kidney sections from the animals in each group.

Extensive periportal necrosis was found in the liver after [^{14}C]AA administration, but no necrosis was observed in the kidneys or lung. After 8 hr, some 120 nmols [^{14}C]AA/g protein was bound in the periportal zone of the liver, and about half that amount was found after 24 hr. Pretreatment with pyrazole reduced the amount of bound radioactivity by 80%, and completely prevented hepatic necrosis. However, microsomal-enzyme induction by phenobarbitone pretreatment had no significant effect on the level of bound radioactivity and had no influence on the hepatotoxicity of AA.

Most of the initial *in vivo* oxidation of alcohols occurs in the liver, and thus little [^{14}C]AA was found in the kidneys and lungs. In turn, this suggests that the degree of tissue necrosis is determined by the extent of covalent binding, as no pathological effects were observed in the two latter organs. The findings in this study thus support the hypothesis that periporta liver necrosis is mediated by an AA metabolite, such as acrolein, the localization of binding and tissue necrosis being determined by the distribution within the liver lobule of the enzyme responsible for the production of this toxic metabolite.

2612. Divergent paths in allyl ester metabolism

Kaye, C. M. & Young, L. (1972). The synthesis of mercapturic acids from allyl compounds in the rat. *Biochem. J.* **127,** 87P.

In rats, the metabolic pathway for various esters of allyl alcohol appears to depend on the strength of the acid from which the ester is derived. Allylmercapturic acid and 3-hydroxypropylmercapturic acid were found in the urine of rats dosed with allyl halides (*Cited in*

F.C.T. 1973, **11,** 686) or with esters of allyl alcohol and strong acids, such as allyl nitrate. On the other hand, only 3-hydroxypropylmercapturic acid was formed in rats given allyl alcohol or one of its esters with weak acids, including formic, propionic, phthalic and nitrous acids.

Two other allyl compounds, allylamine and allyl cyanide, were also found to give rise to urinary 3-hydroxypropylmercapturic acid but not to allylmercapturic acid. The former metabolite, isolated as the dicyclohexylammonium salt, was also found in about 10% yield in the urine of rats dosed with acrolein. By analogy with an earlier finding (Gray & Barnsley, *Xenobiotica* 1971, **1,** 55) suggesting that crotonaldehyde was an intermediate in the conversion of crotyl alcohol to 3-hydroxy-1-methylpropylmercapturic acid, the authors of the paper reviewed here support the view that acrolein is an intermediate in the metabolism of allyl alcohol to mercapturic acid (see previous page). Allylmercapturic acid was not excreted in detectable amounts by rats dosed with acrolein.

EMULSIFIERS AND STABILIZERS

2613. Biochemical study of a soft-drink stabilizer

Procter, B. G., Dussault, P. & Chappel, C. I. (1973). Biochemical effects of sucrose acetate isobutyrate (SAIB) on the liver. *Proc. Soc. exp. Biol. Med.* **142,** 595.

Sucrose acetate isobutyrate (SAIB) is employed in certain countries as a flavour-suspending agent in soft drinks. In November 1972, approval was granted in Brazil for the use of up to 500 ppm of this ester in non-alcoholic drinks, and Canadian regulations permit its use at levels up to 50 ppm. To investigate the safety of this stabilizer for use at rather higher levels than are currently permitted in Canada, SAIB was fed to experimental animals in short-term studies, and the present report describes the effect on several liver-function parameters monitored throughout the studies, including serum levels of glutamic–pyruvic and glutamic–oxalacetic transaminases, alkaline phosphatase (AlkP), lactate dehydrogenase and ornithine carbamoyl transferase.

In the case of beagle dogs, SAIB was fed for 12 wk at dietary levels of 0, 0·5, 1, 2 and 4%. Increased bromsulphthalein (BSP) retention was observed in all SAIB-treated animals throughout the study, but was found to be rapidly reversible on removal of SAIB from the diet. Other blood parameters examined were within normal limits, with the exception of serum AlkP, increases in which were proportional to the duration of SAIB administration as well as to the dose level. These findings were considered to indicate that the primary effect of SAIB was a mild cholestasis. Support for this view was provided by changes in biliary morphology seen under the electron microscope and by increased enzyme activity in the bile canaliculi demonstrated histochemically (Procter *et al. Toxic. appl. Pharmac.* 1972, **22,** 328). Liver hypertrophy and associated effects indicative of microsomal-enzyme induction were seen in male beagles, but again were reversed by SAIB withdrawal and were not accompanied by any histological evidence of toxic change.

When SAIB was fed to albino rats for 12 wk at dietary levels up to 10%, studies of blood biochemistry indicated the absence of any effect on liver function in this species. BSP

clearance studies were not carried out in this case, but separate experiments were undertaken to determine the effect of SAIB on BSP clearance both in rats fed 4% in the diet for 7 days and in squirrel monkeys given a single dose of approximately 2 g/kg. In neither case was any effect detected.

2614. Carrageenan cytotoxicity and the primary immune response

Bice, D. E., Gruwell, D. G., Salvaggio, J. E. & Hoffmann, E. O. (1972). Suppression of primary immunization by carrageenan—a macrophage toxic agent. *Immun. Commun.* **1,** 615.

Among the biological effects of carrageenan (*Cited in F.C.T.* 1971, **9,** 561) is an ability to interfere with various aspects of the immune response. It has been shown to inhibit the C'_1 component of complement (Davies, *Immunology* 1965, **8,** 291), to interfere with the establishment of delayed hypersensitivity reactions (Bice *et al. Int. Archs Allergy appl. Immun.* 1971, **41,** 628) and to inhibit the progress of already established hypersensitivity reactions (*Cited in F.C.T.* 1972, **10,** 260). Carrageenan has also been shown to inhibit stimulation of lymphocytes by antigens that need to be processed by macrophages (Lake *et al. J. Immun.* 1971, **107,** 1745), although it has no effect on lymphocyte stimulation that is not macrophage-dependent.

Further light has now been shed on the mechanism by which carrageenan interferes with the immune response. When red blood cells from sheep are injected into mice by the ip or iv route, a serum antibody is formed. However, when mice undergoing a 5-day course of ip carrageenan injections were injected ip with sheep red blood cells on day 4, little or no antibody was elicited. Injection of large doses of carrageenan iv also suppressed the immune response to sheep red blood cells injected ip, but when the antigen was injected iv into the mice given carrageenan by the ip route, a normal antibody response occurred.

The toxicity of carrageenan to macrophages has been established *in vitro* (*Cited in F.C.T.* 1972, **10,** 260), and the suppression of the immune response demonstrated in this study is thought to be a direct result of the cytotoxic effect of carrageenan on the macrophages.

ANTIOXIDANTS

2615. Two more BHT metabolites

Shaw, Y.-S. & Chen, C. (1972). Ring hydroxylation of di-t-butylhydroxytoluene by rat liver microsomal preparations. *Biochem. J.* **128,** 1285.

In the rat the main metabolite of butylated hydroxytoluene (BHT) is BHT-acid, formed by way of the alcohol and aldehyde (*Cited in F.C.T.* 1968, **6,** 533; *ibid,* 1971, **9,** 906). Conversion to the alcohol is under the control of a liver microsomal enzyme, BHT oxidase (Gilbert & Golberg, *Fd Cosmet. Toxicol.* 1967, **5,** 481). The metabolites BHT-dimer and BHT-dimer-quinone have also been identified (*Cited in F.C.T.* 1971, **9,** 906; Akagi & Aoki, *Chem. Pharm. Bull., Tokyo* 1962, **10,** 101), and the present paper reports that the rat microsomal enzymes are capable of converting BHT into two further compounds by means of ring hydroxylation.

When rat-liver microsomal preparations were incubated with BHT in an NADPH-generating system composed of $NADP^+$, glucose 6-phosphate and glucose-6-phosphate dehydrogenase at 18°C and pH 7·4, formation of 4-hydroxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dienone (I) and its presumed precursor, 4-hydroperoxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dienone (II) reached a maximum within 5–10 min. The formation of BHT-alcohol, BHT-dimer and BHT-dimer-quinone was also confirmed. Production of I, II and BHT-alcohol required the presence of oxygen and was inhibited by carbon monoxide and by SKF 525-A, indicating dependence on a microsomal oxygenase. Boiled microsomal preparations yielded small amounts of oxygenated products, presumably as a result of catalysis by metal ions or complexes. Both compound I and BHT-alcohol were metabolized further, at a more rapid rate than was compound II. Treatment of BHT with *m*-chloroperbenzoic acid also produced compounds I and II and BHT-alcohol, suggesting that their metabolic formation resembled a per-acid reaction and involved an enzyme-oxygen complex containing activated oxygen as -OO- rather than -O-.

[There are strong indications that the metabolic pathway of BHT in man differs from that in the rat, although the situation in man is still under debate (Daniel & Gage, *Fd Cosmet. Toxicol.* 1971, **9**, 320). The relevance of the above findings to the human situation is therefore uncertain.]

PRESERVATIVES

2616. Uses for diethyl pyrocarbonate in biochemistry

Henderson, R. E. L., Kirkegaard, L. H. & Leonard, N. J. (1973). Reaction of diethyl pyrocarbonate with nucleic acid components: Adenosine-containing nucleotides and dinucleoside phosphates. *Biochim. biophys. Acta* **294**, 356.

The beverage preservative, diethyl pyrocarbonate (DPC), has frequently been used by biochemists as an inhibitor of ribonuclease activity, although it was not known how far this inactivation was related to a direct effect on the enzyme protein and how far to the reaction of DPC with nucleic acid components. A previous abstract (*Cited in F.C.T.* 1967, **5**, 110) referred to the apparent non-reactivity of DPC with either DNA or RNA, as measured by the ability of these nucleic acids to serve as substrates for the corresponding nucleases. By utilizing a sensitive spectrophotometric technique to detect reaction products, however, the authors cited above have obtained support for recent evidence suggesting that the structure of nucleic acid components may be modified by the pyrocarbonate.

Adenosine 5′-phosphate and adenosine 3′-(2′)-phosphate were converted on reaction with DPC into the corresponding 4,5-dicarbethoxyamino-6-*N*-ribofuranosylaminopyrimidine phosphates by cleavage of the imidazole ring. These compounds were precipitated and characterized as the disodium salts. When the synthetic dinucleoside phosphates, adenylyl (3′ → 5′)adenosine, adenylyl(3′ → 5′)uridine and uridylyl(3′ → 5′)adenosine, were reacted with DPC, the adenosine units were again split at the imidazole ring to give rise to *N*-carbethoxy derivatives in high yields. The ultraviolet spectra of these derivatives were found to resemble those predicted by adding the absorbances of the corresponding nucleosides.

While this work reinforces previous reservations about the use of DPC as a nuclease

inhibitor, because it indicates that this ability is accounted for in part by reaction of DPC with nucleic acid components, it may also suggest potential applications for the compound in determinations of nucleic acid structure.

2617. Alkylation by propylene oxide

Lawley, P. D. & Jarman, M. (1972). Alkylation by propylene oxide of deoxyribonucleic acid, adenine, guanosine and deoxyguanylic acid. *Biochem. J.* **126,** 893.

Propylene oxide appears in industry as a by-product in a number of processes, but data on the toxicological properties of this reactive chemical are not extensive. It has also been used as a food fumigant (*Cited in F.C.T.* 1966 **4,** 456 & 607; *ibid* 1967, **5,** 425), although it is less popular in this respect than ethylene oxide. An alkylating agent, it is known to be mutagenic for Drosophila, but its possible effects on chromosome structure in animals do not appear to have been investigated in detail. The main *in vivo* target for alkylation by this epoxide would be expected to be DNA, and the present work was carried out to investigate the possible reaction between propylene oxide and this nucleic acid, the limited earlier studies in this connexion having produced somewhat inconclusive results.

The oxide was shown to react to some extent with DNA in aqueous buffer solution at roughly neutral pH to yield two main products, identified by their ultraviolet absorption spectra as 7-(2-hydroxypropyl)guanine and 3-(2-hydroxypropyl)adenine after hydrolysis of the alkylated DNA.

The same products were separated and isolated after reaction of propylene oxide with guanosine and adenine, respectively. The latter reaction, in acetic acid, closely resembled that between dimethyl sulphate and adenine in neutral solution, the degree of reactivity of the alkylated positions in adenine being in both cases N—3 > N—1 > N—9. In the case of the propylene oxide/guanosine reaction in neutral solution, alkylation was apparently confined to the N—7 position on the base.

Mass spectrometric analysis of the major alkylation products led to the suggestion that propylene oxide reacts largely through operation of a bimolecular S_N2 alkylation mechanism. The possibility of S_N1-type reactivity could not be completely eliminated, however, in view of the limitations of the techniques used.

MISCELLANEOUS DIRECT ADDITIVES

2618. Evaluation of FPC

Newberne, P. M., Glaser, O., Friedman, L. & Stillings, B. (1973). Safety evaluation of fish protein concentrate over five generations of rats. *Toxic. appl. Pharmac.* **24,** 133.

Fish meal has been used in animal feeds for a number of years as a valuable source of high-quality protein, and the needs of an increasing world population have led to the development of a fish-protein concentrate (FPC) for human consumption. This greyish, tasteless and odourless powder can be obtained from species which would normally have little economic value, and 10 g FPC has been estimated to supply the minimum daily animal-protein requirements of the average adult. Thus the present study was carried out to demonstrate the safety and wholesomeness of FPC prepared from red hake.

Five generations of rats were fed throughout their lives on a balanced diet containing FPC or casein as the sole source of protein, which constituted 20% of the diet in each case. The test groups of the early generations received FPC containing 0·2–2·0% isopropanol residues, while the FPC given to the last two generations contained only 0·01–0·02% isopropanol. Body and organ weights, feed efficiency, reproduction studies and pathology were studied and offspring were examined for congenital abnormalities. No consistent differences were found between the group fed the semi-synthetic FPC diet and those given the casein diet or a laboratory chow diet in any of the generations. The animals on a normal chow diet showed better reproduction but decreased feed efficiency compared wth those on FPC or casein diets, but the authors relate this difference to a tendency commonly found in animals fed diets based on natural products.

On the basis of the results obtained, the product itself was assessed as being safe, wholesome and nutritious, although only the findings up to the F_3 generation are reported in this paper. The authors draw attention, however, to another possible problem, namely that associated with the concentration of undesirable residues, such as mercury, that may be present in the fish. Analysis of the FPC used in the study showed a mean mercury level of 0·22 ppm, mainly as methylmercury, resulting in a total mercury consumption of about 12 μg/kg/wk. This would be equivalent to some 840 μg/wk for an adult man, an intake that would be provided by 1·85 lb fish containing 1 ppm mercury, which is twice the FDA action level.

[The concomitant concentration of undesirable residues present in the starting material is a possibility that must always be borne in mind in connexion with the preparation of protein concentrates from fish or any other food source.]

AGRICULTURAL CHEMICALS

2619. Metabolism of zineb in the rat

Truhaut, R., Fujita, M., Lich, N. P. et Chaigneau, M. (1973). Etude des transformations métaboliques du zinèbe (éthylènebisdithiocarbamate de zinc) chez le rat. *C.r. hebd. Séanc. Acad. Sci., Paris* (*D*) **276,** 229.

Some attention has been focused on the comparative metabolism of pesticides in plants and animals, following a suggestion that if the same breakdown products were shown to be produced in comparable quantity in plants and animals, the plant metabolites, which would ultimately be ingested by the consumer in the form of residues, need not be subjected to as complete a toxicological examination as the original pesticide. In this connexion, the present study was undertaken on zineb (zinc ethylenebisdithiocarbamate), one of the dithiocarbamate fungicides used to treat fruit and vegetables.

Purified zineb was administered to rats by gastric intubation for 2 wk in six doses of 1 g/kg (3 doses/wk). Carbon disulphide was identified in the exhaled air 2 hr after administration of zineb. Four metabolites were detected in the urine by ultraviolet spectroscopy and the two major ones were subsequently identified as ethylene bisisothiocyanate sulphide and ethylene thiourea (ETU). According to Lyman (*Pure appl. Chem.* 1971, p. 243), ETU undergoes further metabolism in the rat to ethylene urea and ethylenediamine, the latter being excreted mainly as the *N*-acetyl derivative. In plants treated with mancozeb (man-

ganese ethylenebisdithiocarbamate), metabolism to ethylenediamine was followed by further breakdown of the metabolite and ultimate incorporation of the fragments into the normal metabolic pool. However, in view of the fact that ETU has been implicated as a carcinogen (*Food Chemical News* 1971, **13** (26), 17), long-term experiments are being undertaken by the authors cited above to establish more precisely the residual level of ETU that would occur in food intended for human consumption.

THE CHEMICAL ENVIRONMENT

2620. Fibreglass in the clear

Hill, J. W., Whitehead, W. S., Cameron, J. D. & Hedgecock, G. A. (1973). Glass fibres: absence of pulmonary hazard in production workers. *Br. J. ind. Med.* **30,** 174.

Irritation of the upper respiratory tract is often reported by workers handling fibreglass (*Cited in F.C.T.* 1971, **9,** 449), but a study made in Pittsburgh indicated that glass fibres 9–15 μm in diameter did not constitute a health hazard (*ibid* 1972, **10,** 266).

The present paper describes an investigation of 70 workers who had been exposed to the sawing and trimming of fibreglass for 12–24 yr. These workers were compared with a group of matched controls, who came from the same town but who worked in glass warehouses where fibreglass was not used. Lung function, as measured by the Wright peak flow meter, forced expiratory volume in 1 sec and forced vital capacity (FVC), revealed no impairment among the workers; indeed, the FVC showed a slight difference to the disadvantage of the controls. The X-ray findings did not differ significantly between the two groups. Subjective reports of phlegm and rashes were examined, and it appeared that 45% of fibreglass workers had suffered for a short period from a rash. Reports of phlegm by 12 fibre handlers and 19 controls did not indicate any significant difference.

This study therefore reinforced earlier findings and produced no evidence of any respiratory hazard to workers exposed to glass fibres under present conditions, in which the manufacturing specifications call for a diameter of 0·5–25 μm.

2621. Contrasting pictures with inhaled nickel and cobalt oxides

Wehner, A. P. & Craig, D. K. (1972). Toxicology of inhaled NiO and CoO in Syrian golden hamsters. *Am. ind. Hyg. Ass. J.* **33,** 146.

Nickel and cobalt oxides form water-insoluble dusts with similar physico-chemical properties. The compounds have been classified as substances that display moderate retention when deposited in the lung, with intermediate clearance rates of the order of weeks (ICRP Committee, *Hlth Phys.* 1966, **12,** 173). The present series of investigations, however, illustrates that the supposedly insoluble monoxides of nickel (NiO) and cobalt (CoO) differ in their solubility and clearance characteristics and acute toxicity when inhaled by hamsters.

Although NiO was not acutely toxic at any of the atmospheric concentrations used

(max 184 μg/litre), all the animals exposed for 3 hr/day to a mean concentration of 108 μg CoO/litre died by the end of day 4. Post-mortem examination of the tissues revealed a significant difference from the findings in hamsters exposed to non-fatal doses only in the case of the liver, which carried higher CoO levels in the animals that died. In a further inhalation study, hamsters were exposed for a 3-hr period on 5 days/wk for a total of 60 days to mean concentrations of 27·4 or 46·1 μg CoO/litre. During the first 3 days, when the concentrations used were nearly twice these mean levels, there were several fatalities in the higher-dosage group, but all the other animals survived the test period. Data from these tests suggested that the percentage mortality in the hamster was a function not only of the total integrated dose but also of the dose rate. There was some evidence that hamsters that survived the first few exposures developed some tolerance to inhaled CoO.

None of the hamsters exposed to either NiO or CoO aerosols for up to 3 months (4 hr/day, 5 days/wk), at maximum concentrations over a 4-hr period of 184 μg NiO/litre and 17·2 μg CoO/litre, died from the acute effects of the treatment during the course of the exposures. While 17–20% of the NiO estimated to have been inhaled was found in the lungs of animals killed 3–4 days after the last exposure, the comparable figure for CoO was only 0·3–1·1%. As the particle-size distribution of the two aerosols (1·0–2·5 μm diameter) was similar enough to make it unlikely that the difference was due to different deposition patterns, it was considered probable that this was due to mobilization of CoO by solubilization in body fluids or by the formation of some complex. Essentially all the CoO was eliminated from the lungs within 6 days of termination of exposure, at which stage more than 70% of the NiO originally deposited in the lungs was still retained.

Finally a study was made of the gastro-intestinal absorption and tissue distribution of CoO and NiO following gastric intubation of 5 mg of either oxide suspended in 0·5 ml normal saline. The hamsters were sacrificed 24 hr later. The results obtained with NiO are not described in detail, but less than 0·5% of the administered dose of CoO was absorbed and accounted for in the body tissues. Apart from the amount retained in the lung, there was significantly more CoO in both the gut and skinned carcass 24 hr after inhalation than after gavage.

It is suggested that more detailed experiments on the toxicity and biological behaviour of inhaled NiO and CoO should be carried out in other species, such as the dog.

2622. Vermiculite in the lung

Hunter, B. & Thomson, C. (1973). Evaluation of the tumorigenic potential of vermiculite by intrapleural injection in rats. *Br. J. ind. Med.* **30,** 167.

The hazards arising from the use of asbestos have been recognized for some time (*Cited in F.C.T.* 1968, **6,** 657) and alternative materials have been sought. Vermiculite is the geological name for aluminium-iron-magnesium silicates consisting of laminar flakes which contain microscopic droplets of water. When subjected to sudden high temperature this water vapourizes, forcing the laminae apart and producing light, accordian-like granules. Such exfoliated vermiculite resembles asbestos in its low thermal capacity and high insulation value and is therefore a potential substitute in applications providing thermal insulation, anticondensation control, sound absorption and fire protection. Hence this study was initiated to find out whether vermiculite, like asbestos, induced mesotheliomas when injected into the pleural cavity.

Vermiculite was suspended ultrasonically in physiological saline and was injected intra-

pleurally into 50 rats in a dose of 25 mg in 0·2 ml saline. Comparable groups of negative and positive controls were treated with 0·2 ml physiological saline and with chrysotile asbestos (25 mg in 0·2 ml saline) respectively. All animals were maintained for 104 wk after injection unless death intervened. Survival of the vermiculite-treated animals was similar to that of the saline controls, whereas the survival time of the asbestos-treated rats was curtailed. There was a high incidence of mammary and pituitary tumours in the saline controls and in the vermiculite-treated animals. The lower incidence of these tumours in asbestos-treated animals was probably due to their earlier mortality. All groups showed signs of chronic pleurisy, and the asbestos and vermiculite groups showed pleural adhesions and abscesses. Mesotheliomas were found in 48% of the asbestos group, but although granulomas were found in the vermiculite-treated rats, none of these showed signs of neoplastic activity.

Recent studies have thrown some light on the mechanisms by which asbestos injected into the pleural cavity may induce mesothelioma (*ibid* 1972, **10,** 576). While it seems that the chemical contaminants suggested as possible factors in the carcinogenicity of asbestos are likely also to be present in vermiculite, there is a major difference in the type of tissue response elicited by the two materials. Phagocytosis of particles of chrysotile asbestos results in macrophage death and rupture with release of the particles for further phagocytosis, a cyclic process which is accompanied by progressive fibrosis and which eventually terminates leaving the particles lying free within the fibrous tissue (Allison, *Archs intern. Med.* 1971, **128,** 131). The amorphous vermiculite particles, on the other hand, may be more easily retained within granulomas that do not undergo this cyclic process.

While accepting that administration by intrapleural injection is not strictly relevant to the conditions of exposure in man, the authors of the present paper conclude from this study that the use of vermiculite is likely to involve a lesser degree of hazard than is associated with the use of asbestos.

2623. Triethyllead toxicology

Galzigna, L., Ferraro, M. V., Manani, G. & Viola, A. (1973). Biochemical basis for the toxic effects of triethyl lead. *Br. J. ind. Med.* **30,** 129.

The subject of lead pollution has been one of the most exhaustively analysed toxicological issues of the last decade. Tetraethyllead has been the subject of much study, particularly in connexion with the biochemical basis of its toxic effects (*Cited in F.C.T.* 1972, **10,** 435), and triethyllead (Et_3Pb) the highly toxic metabolite, which may accumulate during chronic exposure to tetraethyllead (*ibid* 1969, **7,** 548), has therefore not escaped attention. The present work is concerned with the *in vitro* and *in vivo* inhibition of cholinesterase by Et_3Pb and with the lead compound's interference with cholinergic transmission mechanisms.

The inhibition of cholinesterase activity by various concentrations of Et_3Pb chloride was studied *in vitro* in rat-diaphragm homogenates and was found to be of a non-competitive type. The greatest inhibition obtained was about 25% of the normal activity, with Et_3Pb concentrations of 15–30 mM. A similar degree of inhibition of serum cholinesterase was reached after 10–20 min in dogs given a fast-flowing iv infusion of 6 mg Et_3Pb/kg. Dramatic increases in the duration of succinylcholine-induced myoneural inhibition were also observed, but the administration of pyridine-2-aldoxime methiodide was shown to reverse both these changes. *In vitro*, however, Et_3Pb catalysed the rearrangement of catecholamines to aminochromes and inhibited the effect of catecholamines on smooth muscle contraction.

It was concluded that the toxicity of Et_3Pb must be explained in terms of an interference with both cholinergic and adrenergic transmission mechanisms. The authors suggest that such effects lead to the formation of a psychotogenic complex which is particularly active at the level of the central nervous system.

2624. A toxicological investigation of cyclohexenone

Levin L., Meyers, G. B. & Liddane, Lillian (1972). 2-Cyclohexen-1-one: Toxicology and study of an occupational dermal exposure. *Am. ind. Hyg. Ass. J.* **33,** 338.

2-Cyclohexen-1-one, a compound with a high degree of reactivity, finds potential application in various chemical syntheses. The recent development of a process for producing large quantities of very pure cyclohexenone has opened up opportunities for more technical and research applications, a prospect which prompted the reported toxicological investigations.

Acute toxicity studies demonstrated that the compound was an active primary irritant to the eyes and skin and was highly toxic dermally, the LD_{50} in rabbits being 70 mg/kg. The chronic convulsions and ascending paralysis that followed dermal application of a lethal dose indicated central nervous system involvement. The compound was also toxic when given orally, the LD_{50} in rats being 220 mg/kg, and when inhaled as an aerosol or vapour. The 4-hr LC_{50} of the vapour in rats was determined as 250 ppm.

To study the effects that might be produced by frequent applications of sublethal amounts of cyclohexenone to the skin, a 90-day study in rabbits was initiated. After application of a dose equivalent to 10% of the maximum LD_0 to the intact skin on 5 days/wk for a 60-day period, the animals showed evidence of skin changes (inflammatory cell infiltration and hyperkeratosis) but gross and microscopic examination revealed no evidence of systemic effects or of any significant morphological changes in any of the major organs. For the final 30 days, therefore, the daily dose applied was increased to 10% of the acute dermal LD_{50}, but at the end of the test period there was still no evidence of systemic toxicity, although the animals showed marked epidermal thickening and necrosis, collagen degeneration, inflammatory cell infiltration and local haemorrhage in the skin.

In a further investigation, rabbits showed no significant skin damage, no systemic effects and no demonstrable effects on health at any time during a 7-day observation period following the application of a lethal dermal dose of cyclohexenone which was washed off the skin after only 1 min.

On the basis of these findings, a hygienic standard of 5 ppm was adopted for occupational exposure to cyclohexenone and it was considered that proper eye and skin protection should be mandatory for workers involved with this chemical. It was judged that dermal complications resulting from skin contact without adequate and prompt washing would probably be the major health problem.

The accuracy of this prediction was indicated by the case history of a laboratory worker who delayed washing a skin area contaminated with cyclohexenone and who subsequently developed dermatitis, which took some 6 wk to heal. No systemic effects could be inferred from the man's medical record. No dermatitis or other adverse effects had been seen among other laboratory workers who had had minor skin contact with cyclohexenone but who had immediately washed the skin thoroughly.

2625. Chlorinated methanes against hypertension

Loyke, H. F. (1973). Methylene chloride and chronic renal hypertension. *Archs Path.* **95,** 130.

Methylene chloride (CH_2Cl_2) is usually regarded as one of the less toxic halogenated hydrocarbons, although it has been reported to be a weak nephrotoxin in mice (*Cited in F.C.T.* 1966 **4,** 112). Renal hypertension has been found to be reduced by carbon tetrachloride (CCl_4) and by chloroform ($CHCl_3$) in the rat (Loyke, *Proc. Soc. exp. Biol. Med.* 1964, **115,** 1035; *idem, Anesth. Analg.* 1971, **50,** 825), and the author therefore decided to investigate these properties in CH_2Cl_2.

Hypertension was produced in rats by ligation of both poles of one kidney followed 1 wk later by contralateral nephrectomy. The rats were used when they had maintained a systolic blood pressure above 170 mm Hg for 3 months. Eleven renal hypertensive and three normotensive animals were given up to 15 biweekly sc injections of 1·5 ml CH_2Cl_2/kg. A positive control was provided by two untreated hypertensive rats, and the negative control by 11 normotensive and untreated animals. The rats were killed at wk 24 or 28, and kidney, liver, adrenal and lung tissues were fixed, stained and examined histologically.

Like CCl_4 and $CHCl_3$, CH_2Cl_2 was found to reduce the systolic blood pressure in hypertensive rats, an effect which was reversed when the injections were discontinued. No histological changes were found in the untreated hypertensive animals but the CH_2Cl_2-treated hypertensive animals showed signs of slight liver degeneration. The same group showed slight to moderate degrees of interstitial lymphocyte infiltration in the lung tissues, but otherwise none of the groups differed histologically from the negative control group.

Thus, CH_2Cl_2, like the more toxic chloromethanes, can reduce experimental hypertension in the rat.

2626. Support for the free-radical metabolite theory of halomethane toxicity

Reynolds, E. S. (1972). Comparison of early injury to liver endoplasmic reticulum by halomethanes, hexachloroethane, benzene, toluene, bromobenzene, ethionine, thioacetamide and dimethylnitrosamine. *Biochem. Pharmac.* **21,** 2555.

Perhaps the theory most favoured at present in connexion with the mechanism of halomethane hepatotoxicity is that their free-radical metabolites initiate a peroxidative decomposition of liver microsomal lipids (*Cited in F.C.T.* 1970, **8,** 88). In support of this theory it has been established, at least in the case of carbon tetrachloride (CCl_4), that the endoplasmic reticulum is the first hepatic organelle to exhibit manifestations of intoxication (*ibid* 1970, **8,** 89). Since the reactivity of halomethanes in homolytic cleavage is directly proportional to the extent of halogenation, one way of testing the above hypothesis would be to look for a direct correlation between this reactivity and the extent of damage to the reticular membrane. With this in mind, the author cited above compared the *in vivo* effects of a range of halogenated hydrocarbons (CH_2Cl_2, $CHCl_3$, CCl_4, C_2HCl_5, C_2Cl_6, CH_2Br_2, $CHBr_3$, CBr_4, CH_2I_2, CHI_3 and CI_4) on the function and composition of rat-liver endoplasmic reticulum 2 hr after administration of an oral dose of the compound. Other chemicals tested in a similar fashion were the hepatotoxins, bromobenzene, ethionine, thioacetamide and dimethylnitrosamine (DMNA), and two lipid solvents, benzene and toluene.

In the case of the halomethanes, effects of isomolar doses on the diene-conjugate content of microsomal lipids, on the cell-sap RNA content and on the microsomal enzymes,

oxidative demethylase and glucose 6-phosphatase, increased with decreasing effective negative charge on the halogen atoms (an index of increasing free-radical reactivity). As expected, the most extensive damage was produced by CCl_4 and CHI_3 (iodoform).

Both thioacetamide and DMNA resembled the halomethanes in reducing [^{14}C]glycine incorporation into microsomal protein at 2 hr. With DMNA, the suppression of protein synthesis was accompanied by an enhancement in the diene-conjugate content of microsomal lipids and in cell-sap RNA. However, the hepatotoxins, bromobenzene and ethionine, and the two organic solvents, with solubility properties comparable to those of CCl_4, were without effect on microsomal function 2 hr after their administration, as were the two chloroethanes studied.

The author concludes that his findings are compatible with the hypothesis that halomethanes damage the endoplasmic reticulum through a free-radical reaction. DMNA may have a similar mode of action, but thioacetamide and ethionine appear to exert their toxic effects by some other means.

2627. Haloalkane toxicity in rats and chicks

Nachtomi, Edna & Alumot, Eugenia (1972). Comparison of ethylene dibromide and carbon tetrachloride toxicity in rats and chicks: Blood and liver levels; lipid peroxidation. *Expl mol. Path.* **16,** 71.

As discussed above, the hepatotoxic action of carbon tetrachloride (CCl_4) is generally thought to be initiated by free-radical formation and subsequent peroxidation of hepatic microsomal lipids, a process which in turn triggers various toxic responses. In the wider context of the haloalkanes, the authors cited above provide data to dispute this view, by comparing the pool size and rate of elimination of ethylene dibromide (EDB) and CCl_4 in the blood and liver of rats and chicks together with the levels of diene conjugates in the liver lipids. The latter species was chosen in view of its insensitivity to CCl_4 poisoning.

Although CCl_4- and EDB-pool sizes were similar in rat liver, the elimination rate of EDB was more than four times greater than that of CCl_4, a finding indicative of a much more efficient detoxification mechanism for the former compound in rats. The pool size of EDB in the blood of both the rat and chick was at least five times that of CCl_4, although in each species the elimination rates of the two compounds from the blood were similar. Most prominent were the species differences with regard to the pool size of the liver. Whereas the CCl_4 pool in rat liver was almost twenty times as great as that in chick liver, the relevant EDB pool size in rats was only double that in chicks.

Treatment of rats with EDB induced an appreciable rise in the levels of liver triglyceride within 12 hr, although the rise was somewhat less marked than that previously demonstrated in the case of CCl_4. Although the dibromide is much more toxic to rats than the tetrachloride, conjugated double-bond formation in microsomal liver lipids in rats treated with the former compound was only half that in CCl_4-treated animals. Neither poison induced diene formation in chick-liver lipids, although chicks are as sensitive as rats to the toxicity of EDB.

The authors conclude that the levels of these two compounds in the liver and the rates of their elimination from liver and blood may be primarily responsible for inter-species differences in their hepatotoxic effects. Although it is conceded that diene formation (a result of lipid peroxidation) is one reaction that may occur in microsomal lipids after haloalkane

administration, this study raises doubts about the theory that such conjugation is an aetiological factor in haloalkane toxicity.

2628. Monomeric methacrylates and rat reproduction

Singh, A. R., Lawrence, W. H. & Autian, J. (1972). Embryonic-foetal toxicity and teratogenic effects of a group of methacrylate esters in rats. *J. dent. Res.* **51,** 1632.

Methacrylate resins are widely used for dental prostheses and contact lenses, as well as in a variety of industrial applications. It is said that by 1946, 98% of all dentures contained methyl methacrylate polymers or copolymers. As with most polymers, there is a possibility that small amounts of residual monomer may be present in the polymeric product and may be leached out during use. Allergic reactions to acrylic dentures, for example, have been attributed to the presence of the monomer (*Cited in F.C.T.* 1971, **9,** 145).

The toxicity of methacrylic ester monomers, particularly of the methyl ester, has received considerable attention and it is known that acutely toxic doses given by various routes cause death by respiratory depression. Prolonged inhalation of the methyl ester leads to degenerative changes in the liver, but no pathological lesions were found in dogs fed up to 1000 ppm methyl or ethyl methacrylate in the diet for 2 yr (*ibid* 1964, **2,** 503). However, there has been little information about the embryotoxic or teratogenic effects of these esters, and in this connexion a study now reported compares the effects of several methacrylate monomers and acrylic acid administered ip to pregnant rats.

The three dose levels used were 10, 20 and 33% of the acute ip LD_{50} values, determined as 1·33, 1·22, 2·30, 1·40, 2·47 and 0·02 ml/kg for the methyl, ethyl, *n*-butyl, isobutyl and isodecyl methacrylates and acrylic acid, respectively. Each dose level was given undiluted to groups of five pregnant Sprague–Dawley rats on days 5, 10 and 15 of gestation, and four control groups were given cottonseed oil, distilled water, normal saline or no treatment. The animals were killed on day 20 and a careful study of resorptions, stillbirths, gross malformations, skeletal malformations and foetal size was carried out.

Both acrylic acid and the methacrylate esters evoked a dose-related response in terms of resorptions and gross and skeletal malformations. In comparison with the untreated controls, all the treated groups showed adverse effects in some or all of the parameters studied. However, in an attempt to eliminate effects resulting from the trauma of injection and handling, comparisons were also made with a "volume control" derived from the pooled results of the three treated control groups, which received injections equivalent in volume to the largest doses of the test compounds. On this basis, a significantly increased incidence of resorptions was associated with all three dose levels of ethyl methacrylate, the high doses of the *n*-butyl and isobutyl esters and the two highest dose levels of the isodecyl ester. Gross abnormalities, of which haemangioma was the most common, were increased in all the test groups except those given the low dose of methyl methacrylate or acrylic acid and the two lowest doses of the *n*-butyl ester, but only the group given the top-level dose of acrylic acid showed an incidence of skeletal malformations outside the 95% confidence interval (1·0–14·8%) for the pooled volume control.

Among the methacrylate esters, the isodecyl proved the most toxic to the early embryo, with a high incidence of resorptions, and the methyl and ethyl esters produced the most gross abnormalities. Of the latter two groups, only the ethyl showed skeletal abnormalities, however.

2629. PCP metabolism

Larsen, R. V., Kirsch, L. E., Shaw, S. M., Christian, J. E. & Born, G. S. (1972). Excretion and tissue distribution of uniformly labelled ^{14}C-pentachlorophenol in rats. *J. pharm. Sci.* **61,** 2004.

Pentachlorophenol (PCP) and its water-soluble derivatives are used as fungicides and bactericides in a wide range of uses, from the processing of starches leather, paints and cellulose products to the anti-termite treatment of new wood. The hazards of handling PCP have been pointed out by various authorities in the past, and there have been several reports of cases of poisoning due to percutaneous absorption of PCP present in wood-preservative or disinfectant formulations (*Cited in F.C.T.* 1965, **3,** 845; *ibid* 1971, **9,** 601).

In the study now reported, the excretion and distribution of the material were studied. This aspect has not received a lot of attention, although early studies indicated that the urine was the main route of excretion (Deichmann *et al. J. Pharmac. exp. Ther.* 1942, **76,** 104) and suggested the possibility of cumulative toxicity (McGavack *et al. J. ind. Hyg. Toxicol.* 1941, **23,** 239). Female rats were kept in metabolism cages after receiving an oral dose of [^{14}C]PCP (37–41 mg/kg; specific radioactivity 0·27 μCi/mg). Urine samples were collected at 4-, 6- and 12-hr intervals on days 1, 2 and 3, respectively, and then daily for 7 more days. Faeces were collected daily and the pooled 10-day output for each animal was analysed. Tissue distribution following an oral dose of 31–40 mg/kg was studied in male and female rats kept in metabolism cages for 40 hr after treatment and then killed by decapitation, the lungs, liver, heart, kidneys, adrenals, brain, spleen, muscle, testes, ovaries, stomach, intestines and abdominal fat being analysed for radioactivity.

In the metabolic study, negligible radioactivity was found in the respired air. The greatest urinary excretion occurred between 16 and 20 hr after treatment, and a total of 68% of the administered activity was found in the urine after 10 days. The faeces accounted for 9–13% of the administered dose. The results indicated a two-component pattern of urinary excretion, the half-life of the first component being 10 hr and that of the second 102 days.

The residual radioactivity was uniformly distributed among most of the tissues examined, with higher levels in the kidney and liver. The latter levels could be attributed to the process of excretion via the kidney and, in the case of the liver, to detoxification and/or conjugation prior to elimination. More than 99% of the activity in the blood was in the serum.

2630. More on propylene glycol dinitrate

Jones, R. A., Strickland, J. A. & Siegel, J. (1972). Toxicity of propylene glycol 1,2-dinitrate in experimental animals. *Toxic. appl. Pharmac.* **22,** 128.

Since the suspicion a decade or so ago that sudden deaths among a group of ammunition workers were due to exposure to ethylene glycol dinitrate (EGDN), the biological properties of this compound have been examined in several laboratories (Clark & Litchfield, *Br. J. ind. Med.* 1969, **26,** 150). A potential substitute for EGDN in the manufacture of antifreeze dynamite is the corresponding propylene glycol ester, propylene glycol 1,2-dinitrate (PGDN), but the latter seems to have been subjected to only one major toxicity study (*Cited in F.C.T.* 1970, **8,** 111). The authors cited above aimed to supplement the findings of that study, by examining the toxicity of PGDN in a range of animal species. Because of the type of exposure encountered in industry and the lack of relevant data, they were particularly concerned with the effects of inhalation.

Preliminary acute studies showed the oral LD_{50} of PGDN in rats to be around 860 mg/kg. In an ocular irritation study in which 0·1 ml PGDN was instilled into one eye of albino rabbits, reversible inflammation of the conjunctival tissue was noted after 5 min, but the iris and cornea were not affected. A modified Draize technique was used to study the dermal toxicity and skin absorption of PGDN over a period of 20 days. With doses of 1 g/kg applied for 2 hr and then wiped off, there was evidence of slight erythema, and with 2 g/kg the animals showed signs of weakness and cyanosis. Evidence for skin absorption of the nitrate ester included decreased haemoglobin and haematocrit levels, loss in body weight and death after several daily applications of PGDN in doses of 4 g/kg. Histopathological examination at autopsy revealed degenerative changes in the liver, vacuolar changes in the epithelium of the proximal convoluted tubules of the kidney and some degeneration of myocardial fibres.

Rats, guinea-pigs, rabbits, squirrel monkeys and dogs were exposed to various concentrations of PGDN vapour in acute, repeated and continuous inhalation experiments. Changes attributed to continuous exposure to 67, 108 or 236 mg/m^3 included varying degrees of anaemia, iron-pigment deposition in various tissues, fatty changes in the liver, methaemoglobin formation and markedly increased serum and urinary levels of inorganic nitrate.

Single iv administration of 0·041 g PGDN/kg or more to rhesus monkeys caused emesis, ataxia and apnoea, a dose of 0·41 g/kg proving lethal. In a 90-day behavioural study in this species, monkeys trained to perform in visual discrimination or visual acuity threshold tests were exposed continuously to atmospheric concentrations of 262 mg PGDN/m^3. Weekly behavioural tests were carried out, but no changes were observed in avoidance-behaviour patterns. The only pharmacological effect noted during this study was a dilation of the pupils.

2631. Favourable results on a hydrogenated terphenyl coolant

Henderson, J. S. & Weeks, J. L. (1973). A study of the carcinogenicity for skin of a polyphenyl coolant. *Ind. Med. Surg.* **42** (2), 10.

The hydrogenated terphenyl investigated in this study is used as a coolant for organic reactors in nuclear research programmes. During such use, the coolant may change considerably as a result of being subjected to heat and radiolysis, and it is possible that operators may come into contact with this changed coolant as well as with the starting material. Moreover, since the operating temperature is about 400°C, such exposure may involve both the liquid and vapour phases. These are all factors which must affect the overall toxicological assessment of the coolant, but the study now reported was concerned with the degree of carcinogenicity of the fresh or used hydrogenated terphenyl when applied to the skin of mice and with the possible enhancement of this effect by concomitant exposure to irradiation, another potential hazard of the environment under consideration.

Landsteiner tar was used as a comparative substance, and all materials were applied weekly to the clipped skin of mice, the quantities applied being 50 mg of fresh terphenyl, 80 mg of used coolant and 270 mg of Landsteiner tar.

The first skin tumours appeared after 18 wk in areas treated with tar, and by wk 37 most mice in this group had multiple tumours. The first skin tumour from heated terphenyl (a papilloma) was seen after 33 wk, and tumours had appeared in four of the 26 mice of this group by wk 37. No tumours were seen at wk 37 in the group treated with fresh terphenyl. Experiments in which croton oil application was added to the effect of the heated

coolant after 37 wk demonstrated no marked acceleration in the appearance of tumours. A further experiment indicated that skin irradiation with 255 rads did not promote the carcinogenicity of either form of the terphenyl.

Fresh hydrogenated terphenyl appears to be free from carcinogenic properties, and the heated and irradiated form is only mildly carcinogenic in comparison with tar. The hazard of the coolant to workers therefore appears to be very small, but contact with material that has been used in the reactor should be avoided as a precaution.

2632. Metabolic studies and assessment of TCE exposure

Kimmerle, G. & Eben, Anneliese (1973). Metabolism, excretion and toxicology of trichloroethylene after inhalation. 1. Experimental exposure on rats. *Arch. Tox.* **30,** 115.

Kimmerle, G. & Eben, Anneliese (1973). Metabolism, excretion and toxicology of trichloroethylene after inhalation. 2. Experimental human exposure. *Arch. Tox.* **30,** 127.

Müller, G., Spassovski, M. & Henschler, D. (1972). Trichloroethylene exposure and trichloroethylene metabolites in urine and blood. *Arch. Tox.* **29,** 335.

The hepatotoxic and neurotoxic effects of trichloroethylene (TRI) have been explored in some depth, following exposure of experimental animals to fairly high concentrations of this widely used chemical (*Cited in F.C.T.* 1966, **4,** 618). In a study on human volunteers, in which extensive biochemical and neurological tests were carried out, exposure to 200 ppm for a 5-day working week elicited only mild and somewhat inconsistent responses (*ibid* 1971, **9,** 452), but the maximum allowable concentration for workroom atmospheres (threshold limit value) currently varies in different countries from 4 to 100 ppm, apparently as a result of differences in the interpretation of experimental data. Moreover, there has been growing suspicion that one of the metabolites of TRI, trichloroethanol (TCE), is responsible for the toxic effects of the parent compound. The work of Kimmerle & Eben (cited above) was aimed at probing these areas of TRI toxicology in animals and man, and their first report lays emphasis on the metabolic fate of TRI in rats.

In this study, groups of animals were exposed to TRI concentrations ranging from 50 to 3160 ppm for a period of 4 hr, or in a subacute test to concentrations up to 50 ppm for 8 hr/day, 5 days/wk for 14 wk. Analytical methods were developed to measure the levels of TRI and its metabolites, TCE and trichloroacetic acid (TCA), in expired air, blood and urine.

After acute inhalation of TRI at levels up to 330 ppm, TCE and (to a lesser extent) TCA were the major urinary components and were largely excreted during the first 24 hr. With increasing concentrations of TRI, the proportion of TCE excreted increased. Over the 14-wk exposure, the urinary excretion of TCE increased until wk 10 and then decreased gradually. In contrast, however, TCA levels in the urine remained relatively steady throughout, although again with a slight peak at wk 10. TRI was not detectable in the blood in this study, and blood levels of TCE remained relatively stable during the exposure period, with only a slight peak at about wk 10. Chloral hydrate was also found in the blood, with a maximum level at wk 2. No pathological changes were detected in the subacute study, apart from significantly increased relative and absolute liver weights. The fact that liver function tests gave normal results suggested that the liver enlargement might be a manifestation of enhanced hepatic-enzyme activity, though there was no experimental evidence to substantiate this.

In a further study (second paper, cited above), eight volunteers were each exposed to a concentration of about 40 ppm TRI for 4 hr, and four others were exposed to around 50 ppm for 4 hr/day for five successive days. Concentrations of TRI, TCE and TCA were determined in the blood, urine and expired air after various times.

Following acute exposure, blood TRI levels decreased rapidly and were no longer detectable 4 hr after inhalation, but concentrations of TCE decreased very slowly and the metabolite was still detectable at 96 hr. In the urine, the major part of the TCE was excreted within 24 hr, but the maximal amount of TCA was eliminated on days 2 and 3. Repeated inhalations were associated with only slight increases in blood levels of TRI and TCE, and the urinary levels of the metabolite rose to a maximum after a short period and then remained constant. In the case of TCA, however, urinary levels increased continuously throughout the 5-day period and small amounts were detected up to 12 days after the last exposure. Such findings suggested that the pharmacokinetic behaviour of TRI after a single exposure differed from that after repeated exposure. The metabolic fate of TRI also appeared to vary with species, since the metabolites included chloral hydrate in the rat but not apparently in man.

The authors of the third report cited describe a new method to establish a quantitative relationship between TCI exposure levels and the levels of metabolites in the urine and blood. Previous attempts to monitor TRI exposure had utilized the 24-hr total TCA and TCE in the urine as an index, but this introduced problems since the two metabolites did not exhibit identical pharmacokinetic behaviour. It is now suggested that a more reliable index of preceding overall exposure to TRI could be based on the simultaneous determination of the individual metabolites in the blood, using a gas chromatographic (GLC) micro-technique.

After daily exposure of five volunteers to 50 ppm TRI for 6 hr, plasma levels of TCA, which binds readily to plasma proteins, increased almost linearly up to the fifth and final day of exposure, reaching a concentration of about 50 μg/ml. The increase in TCE, however, was more irregular, the highest level obtained being 2·3 μg/ml, as determined by the GLC technique.

2633. Long-term exposure to vinyl chloride

Jühe, Susanne u. Lange, C.-E. (1972). Sklerodermieartige Hautveränderungen, Raynaud-Syndrom und Akroosteolysen bei Arbeitern der PVC-herstellenden Industrie. *Dt. med. Wschr.* **97,** 1922.

Exposure to about 300 ppm vinyl chloride may have an adverse effect on liver function (*Cited in F.C.T.* 1973, **11,** 518), and under certain circumstances industrial exposure to vinyl chloride may be followed by softening of the bones of the extremities, by neurological and circulatory disturbances and by liver enlargement.

The paper cited above describes seven patients who reported symptoms of illness after working for 1–3·5 yr on polymerization processes for PVC production. Their symptoms included hypersensitivity to cold, paraesthesia, deafness, blanching of the fingers, pressure-pain in the finger-tips and loss of grip. Later, swelling of the terminal finger joints and changes in the nails developed. In single cases, failure of vision, abdominal pain or giddiness was reported. Five patients developed Raynaud's syndrome, five a drumstick-type thickening of the finger-tips and prominent hour-glass deformation of the nails, and five had swollen fingers with nodular infiltration of the finger webs, the back of the hand and the

region distal to the elbow. Banded osteolysis of the terminal phalanges of the fingers appeared in five of the seven.

Clinical tests showed indications of liver dysfunction in two of the patients and obstructive respiratory disease in one. Three patients showed skin changes characteristic of the early inflammatory stage of a progressive scleroderma. So far, the pathogenesis of this array of effects remains obscure.

NATURAL PRODUCTS

2634. Vitamin B_{12} absorption reduced by tannic acid

Carrera, G., Mitjavila, S. et Derache, R. (1973). Effet de l'acide tannique sur l'absorption de la vitamine B_{12} chez le rat. *C.r. hebd. Séanc. Acad. Sci., Paris (D)* **276,** 239.

Large doses of tannic acid reduce the intestinal absorption of certain substances such as glucose and methionine, a phenomenon which may be due to the formation of insoluble complexes or to a direct action on the gut epithelium (*Cited in F.C.T.* 1972, **10,** 733). The present study shows that vitamin B_{12} absorption, too, can be reduced by tannic acid.

Rats were given tannic acid intragastrically at dose levels of 250, 500 or 1000 mg/kg, followed 2 hr later by 0·25 μg vitamin B_{12} labelled with ^{57}Co, and the faeces were collected each day for 7 days. At all tannic acid dose levels, faecal weight was significantly decreased during the first 24 hr, suggesting an initial slowing-down of gut movement. In the next 24 hr there was a slight increase in faecal weight, accompanied by a large increase in faecal elimination of vitamin B_{12}. This increase was particularly marked at the highest dose level. However, even at this dose level some vitamin B_{12} was apparently absorbed by the animals. In a study to be published shortly the same team has shown that this effect is due to the formation of a non-absorbable complex of tannic acid, vitamin B_{12} and glycoproteins in the gastro-intestinal tract.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2635. Tracing the sensitizer in alkyl ethoxysulphate

Walker, A. P., Ashforth, G. K., Davies, R. E., Newman, E. A. & Ritz, H. L. (1973). Some characteristics of the sensitizer in alkyl ethoxy sulphate. *Acta derm.-vener., Stockh.* **53,** 141.

An outbreak of contact hypersensitivity among users of a liquid dish-washing product in Norway during 1966 was attributed to its content of alkyl ethoxysulphate (AES), in which the alkyl chain length was predominantly C_{12} (Magnusson & Gilje, *Acta derm.-vener., Stockh.* 1973, **53,** 136). Since clinical testing of some 70,000 women with more than 1500 batches of AES produced no allergic reactions, and since there had been no evidence of sensitization following the widespread use of AES-containing products elsewhere, it was suspected that the Norwegian outbreak was due to a contaminant unique to a particular batch of AES. This has now been confirmed by the present study.

In the guinea-pig maximization test (Magnusson & Kligman, *J. invest. Derm.* 1969, **52,** 268), a 0·5% solution of AES from the batch responsible for the Norwegian outbreak

induced a 71% incidence of sensitization. However, none of the animals sensitized in this way gave a positive response to challenge doses of AES from a batch recently produced by the same factory or to AES from 17 other batches representing a variety of manufacturing locations, raw-material sources, process conditions and alkyl chain lengths. Attempts to sensitize guinea-pigs with some of these other batches also failed. Re-challenge of the sensitized animals with the original material again produced a significant incidence of reactions, indicating that the animals had not lost their sensitivity over the 3-month course of the experiments. Petroleum-ether extracts of the original AES batch were capable not only of provoking a response in previously sensitized animals but also of inducing sensitization. The residue left after extraction accounted for more than 99% of the AES. It elicited a low incidence of positive reactions after only brief extraction, but prolonged extraction rendered it completely inactive. Guinea-pigs tested by the method of Buehler (*Archs Derm.* 1965, **91,** 171) also responded both to the original AES and to its extract, but negative results were obtained with both test methods on the extracts and residues of AES from other sources.

Since AES is highly polar and cannot be extracted to any appreciable extent by petroleum ether, these results suggest that the active sensitizer was relatively non-polar and could be completely removed from AES by prolonged extraction. Work is now in progress in several laboratories to identify this allergenic factor and to define the conditions under which it can and cannot be produced.

2636. Sensitivity to benzalkonium chloride

Fisher, A. A. & Stillman, M. A. (1972). Allergic contact sensitivity to benzalkonium chloride: Cutaneous, ophthalmic, and general medical implications. *Archs Derm.* **106,** 169.

Last year (*Cited in F.C.T.* 1973, **11,** 167) we reported on a case of contact dermatitis, which was ascribed to cetalkonium chloride in a deodorant, since the patient reacted positively when tested either with benzalkonium chloride (BAK), a closely related quaternary ammonium compound, or with the deodorant itself. BAK (alkylbenzyldimethylammonium chloride) is itself widely used as a preservative in ophthalmic preparations (especially in solutions for contact lenses) and in deodorants and cosmetics, and as a disinfectant for the skin and for surgical instruments. It is also used industrially in the fabrication of textiles and dyes.

There have been a few reports of allergic contact dermatitis produced by BAK, mainly in medical personnel exposed to instruments soaked in the antiseptic. A case has now been reported of allergic sensitivity to BAK present in two ophthalmic solutions, which had been applied in succession to the patient's eyes and caused oedema and conjunctivitis. The BAK concentrations in the two solutions were 0·004 and 0·01%. The second preparation was applied in an attempt to clear up conjunctivitis unknowingly caused by a reaction to the BAK in the first formulation. It must be noted that the patient had a history of atopic dermatitis and inhalant sensitivities.

The authors point out that patch-testing with ophthalmic preparations presents particular problems, because such preparations often include prednisolone, a corticosteroid which may suppress the patch-test reaction, or disodium edetate, which may itself evoke a sensitivity reaction. It is therefore important that individual components are tested separately.

2637. Mercury poisoning from cosmetics

Marzulli, F. N. & Brown, D. W. C. (1972). Potential systemic hazards of topically applied mercurials. *J. Soc. cosmet. Chem.* **23,** 875.

Use of skin-bleach creams containing ammoniated mercury (AMM) may lead to elevated blood mercury levels (Frithz, *Acta derm.-vener., Stockh.* 1970, **50,** 345) and in time to nephrosis (Silverberg *et al. Archs intern. Med.* 1967, **120,** 581; Williams & Bridge, *Lancet* 1968, **ii,** 602), to pink disease (Ward & Hingerty, *J. Ir. med. Ass.* 1967, **60,** 94) or to polyneuropathy (Ross, *J. Am. med. Ass.* 1964, **188,** 830). Marzulli & Brown (cited above) have investigated the skin-penetrating ability of AMM and of phenyl mercuric acid (PMA), which is used as a preservative in some topical cosmetics, and have described six more cases of mercury poisoning resulting from prolonged use of AMM-containing creams.

Stratum corneum excised from the human forearm was used to measure the penetration rate of ^{203}Hg-labelled PMA and AMM, the former at concentrations of 0·001–1·00% in 95% alcohol and the latter at 3·9–9·9% in cream (compared with normal use levels of 0·0006–0·05% for PMA and 1–5% for AMM). PMA proved to be poorly absorbed, with a mean penetration rate over 24 hr of 0·2–5·0 ng/cm^2/hr at concentrations of 0·06–1·0% and with virtually zero penetration at 0·001%. AMM, on the other hand, showed evidence of considerable absorption, which appeared to be inversely related to concentration, the penetration rate during the first 24 hr being 200 ng/cm^2/hr at 3·9% but only 50 ng/cm^2/hr at 9·9%. A similar inverse relation between concentration and absorption has also been demonstrated with phenol. The penetration rate of both PMA and AMM decreased in the period between 24 and 72 hr, probably because of their reaction with the skin tissue.

Data were then collected on six women who had used skin-bleach creams containing 1–3% AMM for 2 yr or more. All six displayed symptoms of mercurialism including numbness and aching of the extremities, weakness and ataxia, and some also suffered from nervousness, difficulties of speech, vision or hearing, or urinary incontinence. Urinary 24-hr mercury excretion, normally about 8 μg, was clearly elevated in three cases, at 945, 634 and 251 μg. The last two values were determined many months after use of the cream had been stopped. Whole-blood mercury was as high as 120 ng/ml in one subject and total hair mercury was also raised, the highest level reported being 128 ppm. Further studies on one subject showed that the mercury in blood and hair was largley inorganic, indicating that it was derived from AMM rather than from the diet, in which most of the mercury exists in the methyl form.

The authors estimate that at least 2·4 μg mercury/cm^2/day may be absorbed after skin application of 3% AMM cream, leading to an estimated mercury uptake on normal usage at least 20 times greater than that from the normal diet.

[The FDA last year proposed to ban all uses of mercury in cosmetics, except at preservative levels in some eye-care products where no suitable alternatives were available (*FDA Consumer* 1972, **6** (6), 30). In the case of AMM creams, such a move would certainly seem to be supported by this study.]

METHODS FOR ASSESSING TOXICITY

2638. Testing for atopic allergy

Hirsch, S. R. & Zastrow, Joyce E. (1972). Basophil degranulation: A new method of observation and its correlation with skin testing. *J. Allergy clin. Immun.* **50,** 338.

Studies of atopic reactions have produced variable results. Basophil degranulation in response to a specific antigen has been used as the basis of many studies, but the results have shown poor reproducibility because of individual variation between investigators in their assessment of whether degranulation has occurred. Fluorometric determination of histamine release from human leucocytes has therefore been the method of choice, although the basophil degranulation test is simpler.

The present study describes a method for a more objective assessment of the degranulation of basophils obtained from venous blood. Basophil concentrates were incubated with buffer (control) or buffered antigen in a range of concentrations and afterwards stained with toluidine blue in aqueous aluminium sulphate. The non-degranulated cells appeared as very dark bluish-purple cells about 10–20 μm in diameter. Only these cells were counted, and if the decision took more than 10 sec the cell was not included in the count. The procedure was cross-checked by the repeated counting of four slides, ten times each, by three technicians, and the mean coefficient of variation was 4%.

Basophils from 17 ragweed-sensitive patients and from 13 non-atopic patients were treated with different concentrations of ragweed extract and counted, together with control samples. The mean maximal degranulation in the sensitive group was 77%, while that in the non-sensitive subjects was 9%. It is important to recognize, however, the degree of degranulation that may be considered a significant result, since the range of values in the non-atopic group was 0·0–26%. Standard deviation studies on buffer control incubations led to the conclusion that a maximal degranulation of at least 35% had to be obtained if a result was to be considered positive. Specificity of the test was confirmed by the completely negative results obtained by incubating walnut extract with basophils from ragweed-sensitive patients who were not sensitive to walnuts.

Results of basophil tests and skin tests on various concentrations of ragweed extract were roughly parallel, although the end-point was less clear in the skin tests and degranulation was the slightly less sensitive test. Histamine release from leucocytes, the usual means of testing for atopic sensitivity, is a clear indication of IgE-mediated, immunological reactions, but the combination of anti-IgE with basophils causes both histamine release and degranulation. This study, although small, indicated that the degranulation involved was the same as that taking place in histamine-release systems. The authors feel that basophil degranulation is a less subjective method of study than others, although it should be complementary to rather than a replacement for skin tests.

2639. Search for nitrosamine bioassay

Nishie, K., Norred, W. P., Wasserman, A. & Keyl, A. C. (1972). Phototoxicity and differential hepatotoxicity as biological indicators of nitrosamine activity. *Toxic. appl. Pharmac.* **23**, 680.

The knowledge that many nitrosamines are carcinogenic and the accumulating evidence that a number of environmental situations, and particularly the mixtures of compounds present in various foods and animal feeds, may predispose to nitrosamine formation have provoked much activity not only in the biological testing of various nitrosamines but in the development of analytical methods for their detection. Development of a general chemical method for identifying volatile and non-volatile nitrosamines in a given food mixture is hindered by interference from other constituents, and use of the Pekin duckling

toxicity test for screening involves a 30-day feeding trial and considerable quantities of the test feed. With a view to developing more sensitive bioassay procedures, an attempt was made to relate the phototoxic properties of nitrosamines in protozoa or the anabolic and catabolic hepatotoxicity of these compounds to their known carcinogenicity or non-carcinogenicity. Phototoxicity to the protozoan *Tetrahymena pyriformis* proved unsuitable for distinguishing between carcinogenic and non-carcinogenic nitrosamines, since 22 nitrosamines tested all showed some phototoxicity, to an extent apparently related only to the rate of photodecomposition of the nitroso group. Some of the most phototoxic were in fact compounds accepted as being non-carcinogenic (e.g *N*-nitrosodiphenylamine and *N*-nitrosodibenzylamine), a situation in contrast to that reported for polycyclic hydrocarbons, in which carcinogenicity correlated with phototoxicity (Epstein *et al. Nature, Lond.* 1964, **204**, 750).

More promising, however, were the results of the second part of the study. These suggested that the ability of nitrosamines to induce or inhibit liver microsomal enzymes might provide a basis for a sensitive and relatively quick method of differentiating carcinogenic and non-carcinogenic nitrosamines. The biochemical effects of nitrosamines on the liver were monitored by recording the effect of the compounds on pentobarbitone sleeping times (PST) in mice. The nitrosamines known to be carcinogenic were found to prolong PST under specified conditions, while all the known non-carcinogenic nitrosamines studied shortened it.

BIOCHEMICAL PHARMACOLOGY

2640. Strain differences in rat sensitivity to enzyme inducers

Jori, A., Pugliatti, C. & Santini, V. (1972). Differences in microsomal enzyme induction between Sprague–Dawley and Long–Evans rats. *Pharmacology* **7**, 296.

This paper describes the effects of various inducing agents on hepatic microsomal-enzyme activity in two strains of rat, Sprague–Dawley (S–D) and Long–Evans (L–EA). The effect of phenobarbitone (I) was also compared in rats of the latter strain from two different sources (L–EA and L–EB).

Administration of I to S–D and L–EA rats over 3 days in ip doses of 5, 10 or 50 mg/kg/day, increased the quantity of metabolites (*p*-nitrophenol, 4-aminoantipyrine and *p*-aminophenol) formed from *p*-nitroanisole, aminopyrine and aniline, respectively, by a 9000 **g** supernatant fraction of liver homogenate from these rats. The effect was proportional to the dose of I only for S–D rats, and L–EA rats were less responsive at all doses. Three daily doses of DDT (125 mg/kg, ip) or eucalyptol (500 mg/kg, sc) were also less effective in L–EA than in S–D rats, although 20 mg 3-methylcholanthrene/kg injected ip under similar conditions had a similar effect on enzyme induction in both strains.

Following injections of 50 mg I/kg/day for 3 days, greater concentrations of I were found in the plasma, liver and brain of L–EA than of S–D rats at 8, 24 and 48 hr after the third dose. When concentrations of I in the tissues were similar (at 24 and 48 hr, respectively, in S–D and L–EA rats) the L–EA rats were still less sensitive than S–D to the inducing effects of I, as measured by the increase in the metabolites formed from *p*-nitroanisole, aminopyrine and aniline.

Pentobarbitone, injected in an ip dose of 30 mg/kg 24 hr after the last of three injections of 25 mg I/kg, did not induce sleep in the S–D rats but had a significant effect in over 50% of the L–EA rats.

The differences between L–EA and S–D rats were not seen with the L–EB rats, which came from a different source. Basal enzyme activity and I induction, as judged by *N*-demethylation of aminopyrine, were similar in the L–EB and S–D rats and the higher plasma levels of I seen in L–EA rats 48 hr after its administration were not found in the L–EB rats, in which the levels were comparable to those in the S–D strain.

2641. Metabolic influences on biliary excretion rates

Levine, W. G. & Singer, R. W. (1972). Hepatic intracellular distribution of foreign compounds in relation to their biliary excretion. *J. Pharmac. exp. Ther.* **183,** 411.

Levine, W. G. (1972). Biliary excretion of 3-methylcholanthrene as controlled by its metabolism. *J. Pharmac. exp. Ther.* **183,** 420.

It is well-established that the excretion of foreign compounds in the bile is usually preceded by some biotransformation of the parent compound, and in many cases such transformation appears to be the rate-limiting step in the biliary excretion. Two relevant examples are the carcinogens, benzo[*a*]pyrene (BP) and 3-methylcholanthrene (MC), and the steps preceding their elimination in the bile are given detailed consideration in the two papers cited above.

The first report describes the hepatic intracellular distribution of BP and MC after their iv injection into rats in single doses of 2·5 mg/kg. The compounds were taken up rapidly by the liver, and incorporated largely into the heavier membrane fractions (600 and 10,000 g). Subsequent biotransformation resulted in a transient build-up of metabolites in the cytosol fraction, followed by rapid transfer into the blood and bile. Direct addition of BP or MC to liver homogenates resulted in their being bound to the same membrane fractions as *in vivo*, while metabolites added *in vitro* or injected *in vivo* were recovered mainly in the cytosol. Subcellular distribution of these carcinogens and their metabolites therefore seems to be related to their physico-chemical properties rather than to the occurrence of divergent transport mechanisms.

Examination of the behaviour of succinylsulphathiazole and ouabain, two compounds that are excreted into the bile without prior metabolism, showed that they predominated in the cytosol fraction at all times after injection. Transport into the cytosol may therefore be a general prerequisite for biliary excretion.

In the second study, [^{3}H]MC was injected iv in a dose of 2·5 mg/kg into female rats, and much of the administered radioactivity was recovered in the bile, entirely in the form of polar [^{3}H]MC metabolites. Pretreatment with inducers of foreign-compound metabolism (benzo[*a*]pyrene or phenobarbitone) accelerated the rate of metabolite excretion by this route, while prior treatment with inhibitors of drug metabolism (SKF 525A, piperonyl butoxide or metyrapone) induced a significant retardation in the biliary excretion of radioactivity. The appearance of metabolites in the blood was similarly affected by these pretreatments, and the enhancement or suppression of MC metabolism by these agents *in vitro* also paralleled the changes in biliary excretion of metabolites, except in the case of metyrapone, which had only a weak effect on *in vitro* metabolism of MC.

When a mixture of [^{3}H]MC metabolites was injected iv, the biliary excretion of radioactivity was much more rapid than after administration of the tritiated parent compound.

This rate was not affected by inducers or inhibitors of drug metabolism, indicating that these agents directly influenced the biotransformation of [^{3}H]MC rather than the transport of metabolic products into the bile. The rate-limiting step in the biliary excretion of MC thus appears to be its metabolism.

CANCER RESEARCH

2642. Possible biochemical basis for carbon tetrachloride carcinogenicity . . .

Rocchi, P., Prodi, G., Grilli, S. & Ferreri, A. M. (1973). *In vivo* and *in vitro* binding of carbon tetrachloride with nucleic acids and proteins in rat and mouse liver. *Int. J. Cancer* **11,** 419.

Carbon tetrachloride (CCl_4) has recently been shown to be a weak liver carcinogen in several strains of rat (Reuber, *J. natn. Cancer Inst.* 1970, **45,** 1237; Reuber & Glover, *ibid* 1970, **44,** 419), although the mechanism of its carcinogenic action is unknown. It has, however, been demonstrated that CCl_4 can bind to proteins, and it appears that an acylating metabolite of the solvent reacts with the ϵ-amino groups of proteins (Cessi *et al. Biochem. J.* 1966, **101,** 46c). The present work was undertaken to examine the extent of CCl_4 binding to DNA and related macromolecules.

When injected ip, ^{14}C-labelled CCl_4 bound to the DNA in mouse liver and to the hepatic ribosomal RNA of rats when the animals had been pretreated with 3-methylcholanthrene (3-MC). In both animal species, ^{14}C also became incorporated into nuclear and cytoplasmic proteins, the extent of binding being increased in the latter case by pretreatment with 3-MC. In the presence of hepatic microsomes and pH 5 enzymes from rats and mice pretreated with 3-MC, CCl_4 was metabolized *in vitro* to a compound that could react with DNA and polynucleotides.

Since all polynucleotide fractions were labelled, albeit to varying degrees, binding could not be associated with a particular base. Subcellular fractions from mouse liver induced a higher degree of binding than those from the rat, possibly because of a difference in the pattern of enzymes in the respective microsomal fractions.

2643. . . . and for urethane carcinogenicity

Glazer, R. I., Cinti, D. L., Murahata, R. I., Schenkman, J. B. & Sartorelli, A. C. (1972). Metabolic action of urethane on protein synthesis and drug oxidation in normal and regenerating liver. *Biochem. Pharmac.* **21,** 2867.

One of the well-beaten trails of modern toxicology is that in pursuit of the biochemical bases of carcinogenicity. Of the numerous theories put forward to explain the biochemical aetiology of tumour induction, the most popular is the idea that certain carcinogens or their metabolites interact with macromolecules such as nucleic acids and proteins. More specifically, a number of recent reports have described the degranulating effect of these carcinogens on the endoplasmic reticulum of the liver. In the present study, the action of the potent hepatocarcinogen, urethane, on polyribosome-membrane interactions was investigated by the monitoring of protein synthesis and mixed-function oxidase activity at these locations.

Partial hepatectomy markedly increased protein synthesis both on membrane-bound and free polyribosomes. The increase was somewhat greater on the bound polyribosomes, reaching some 350% 12–18 hr after the operation, but overall the rate of synthesis was approximately doubled for up to 36 hr.

Following ip administration of 1 g urethane/kg to sham-operated rats, there was again an increase in protein synthesis on membrane-bound polyribosomes and also to some extent on free polyribosomes, maximum labelling of nascent protein occurring about 12 hr after the urethane injection. Further studies using urethane doses of 0·25–1·0 g/kg showed this response to be dose-dependent. In contrast, injection of the chemical 12 hr after partial hepatectomy triggered no further increase in peptide synthesis on bound polyribosomes and caused only a relatively slight increase on free polyribosomes above that produced by the hepatectomy.

Although aniline hydroxylation was roughly doubled after injection of 1 g urethane/kg to sham-operated rats, the effect on aminopyrine demethylation, the activity of NADPH-linked cytochrome *c* reductase and cytochrome b_5 and *P*-450 levels was slower and much less marked. In partially hepatectomized rats injected with urethane, the induction of aniline hydroxylase was 60–70% lower than that in sham-operated rats and no significant effect on other enzymes was detected.

It was inferred from the above data that, while the mechanism by which the carcinogen, or a metabolite, stimulated nascent protein synthesis remained uncertain, it was unlikely to involve direct interference with the integrity of the polyribosome-membrane complex. The pattern of protein synthesis on membrane-bound polyribosomes following urethane administration suggests that these bound ribosome complexes are of major importance in the synthesis of specific mixed-function oxidases.

2644. Nitrosamine demethylation by mouse DNA

den Engelse, L. & Emmelot, P. (1971/72). Effects of feeding the carcinogen dimethylnitrosamine on its metabolism and methylation of DNA in the mouse. *Chemico-Biol. Interactions* **4**, 321.

A correlation between nucleic acid alkylation by nitrosamine and eventual tumour production in organs susceptible to these carcinogens has been suggested by numerous workers, although the extent to which such a proposed connexion can be regarded as aetiological remains in doubt (*Cited in F.C.T.* 1971, **9**, 442). An important issue here is the extent to which alkylation can be considered to be irreversible, or, more specifically, the way in which nitrosamines control the activity of the enzyme responsible for this step. The present authors examined this enzymatic conversion in rat liver, during and after a period in which dimethylnitrosamine (DMNA) was administered in the drinking-water.

Pretreatment of mice with 10 ppm DMNA in the drinking-water for a 6-wk period induced marked differences in 7-methylguanine formation in the DNA of lung, liver and kidney cells determined 48 hr after ip injection of a single dose of [^{14}C]DMNA, compared with the response to such injection in mice that had not received DMNA pretreatment. Such changes were reversible, however, in that virtually normal levels of DNA methylation were restored when some 10 wk had elapsed between the termination of the pretreatment and the injection, and the effect was largely overcome after only a 2-wk gap. The pretreatment with DMNA caused a strong depression of hepatic DMNA-*N*-demethylating enzyme activity, and also increased the half-life of unchanged DMNA in the blood.

The authors concluded that the effect on liver *N*-demethylating-enzyme activity was the limiting factor in determining the rate of hepatic DNA methylation after DMNA administration to mice. In addition, the level of enzyme activity governed the amount of DMNA available for demethylation in other tissues, notably the lungs and kidneys.

TERATOGENESIS

2645. A different look at anencephaly

Knox, E. G. (1972). Anencephalus and dietary intakes. *Br. J. prev. soc. Med.* **26**, 219.

Elsewhere in this issue (p. 1134), we have discussed the latest round of arguments on the suggested link between the consumption of blighted potatoes in pregnancy and the birth of infants with the neural-tube defects, anencephaly and spina bifida. This paper reports a much broader study of possible associations between neural-tube defects and a wide variety of dietary components, a study prompted by findings of strong social correlations and characteristic geographical and temporal variations in the incidence of such congenital defects. The incidence of anencephaly in England and Wales was assessed from records of stillbirths and infant deaths between 1961 and 1967, and these findings were examined in relation to variations in the consumption of about 77 selected foodstuffs over the same period, allowing a time interval of between 5 and 9 months between food intake and the corresponding anencephaly data. Food consumption statistics were obtained from the quarterly tabulations in the Annual Reports of the Food Survey Committee.

According to the data assembled, consumption levels of bread, cereals, ice-cream, canned peas and some varieties of cooked-meat products demonstrated a positive association with the subsequent occurrence of anencephaly. For various reasons, the apparent correlations with bread, cereals and ice-cream were not considered to be biologically meaningful, and attention centred on the two remaining types of product, each of which contained an almost specific food additive. These additives, magnesium salts used to maintain the green coloration of canned peas and nitrates and nitrites used for the preservation of cured meats, are used in few other food products, and both canned peas and cured meats have geographical distributions of consumption that show some parallel with regional variations in the incidence of anencephaly. No significant association was found, however, in the case of canned haricot beans, which do not require the addition of magnesium salts.

It is suggested that these food additives and associated processes, especially those involved in the curing of meat, should be studied further in this context.

[Suggestions for further study are, as the author points out, the most that can be gained from exercises of this kind. Despite the interest the findings may promote, it is important to bear in mind that the large number of variables involved make firm correlations impossible to establish. The significance of statistical studies unsupported by other types of evidence should not be exaggerated.]

MEETING ANNOUNCEMENT

TOXICITY OF ENVIRONMENTAL CHEMICALS AND DRUGS

The Seventh Saratoga Conference on Molecular Biology and Pathology will be held on 5–9 August 1974, at Saratoga Springs, New York, under the Chairmanship of Dr. Leon Golberg, Research Professor of Pathology and Scientific Director, Institute of Comparative and Human Toxicology, Albany Medical College, Albany, New York.

This annual conference is held in the delightful environment of Saratoga Springs. The past six conferences have been concerned with various aspects of the contribution that molecular biology is making to the understanding of the mechanisms underlying pathological processes, including cancer.

The 1974 Conference will direct its attention to toxic changes brought about by environmental chemicals and drugs. A group of distinguished speakers will present papers for discussion in the fields of mutagenesis, carcinogenesis, the toxicology of polychlorinated compounds, the correlation of pharmacokinetic parameters with morphological changes, and the role of primates in toxicity testing. Future needs for the establishment of safety will also be discussed.

Details of the programme and application forms will be provided on request by Dr. K.-T. Lee, Department of Pathology, Albany Medical College, Albany, New York 12208, USA.

FORTHCOMING PAPERS

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

Short-term toxicity of Violet 6B (FD & C Violet No. 1) in the rat. By I. F. Gaunt, Joan Hardy, Ida S. Kiss and S. D. Gangolli.

Long-term toxicity of Violet 6B (FD&C Violet No. 1) in mice. By P. Grasso, Joan Hardy, I. F. Gaunt, P. L. Mason and A. G. Lloyd.

Effects of butylated hydroxytoluene on the cell cycle and chromosome morphology of phytohaemagglutinin-stimulated leucocyte cultures. By L. J. Sciorra, B. N. Kaufmann and Roberta Maier.

Studies of the acute and long-term oral toxicity of chlorpyrifos (*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate). By Susan B. McCollister. R. J. Kociba, C. G. Humiston, D. D. McCollister and P. J. Gehring.

The effect of polychlorinated biphenyls on rat reproduction. By R. E. Linder, T. B. Gaines and R. D. Kimbrough.

Penicillium viridicatum mycotoxicosis in the rat. I. Ocular lesions. By M. D. McCracken, W. W. Carlton and J. Tuite.

Penicillium viridicatum mycotoxicosis in the rat. II. Scrotal lesions. By M. D. McCracken, W. W. Carlton and J. Tuite.

Penicillium viridicatum mycotoxicosis in the rat. III. Hepatic and gastric lesions. By M. D. McCracken, W. W. Carlton and J. Tuite.

5-Hydroxytryptamine content of bananas and banana products. By G. Vettorazzi. On the pH of tobacco smoke. By K. D. Brunnemann and D. Hoffmann.

Long-term toxicity study of *n*-propyl gallate in mice. By J. C. Dacre. (Short Paper).

Über das Vorkommen von Aflatoxin M in Trockenmilcherzeugnissen. By Maike Jung und E. Hanssen. (Short Paper).

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.

Some other Pergamon Journals which may interest readers of *Food and Cosmetics Toxicology:*

Annals of Occupational Hygiene

Archives of Oral Biology

Atmospheric Environment

Biochemical Pharmacology

Chronic Diseases

European Journal of Cancer

Health Physics

Journal of Aerosol Science

Journal of Neurochemistry

Life Sciences

Toxicon

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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).

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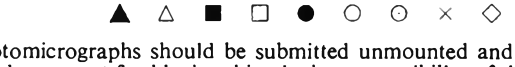

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