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Research Section

EFFECTS OF DIETARY PHOSPHOLIPIDS AND ODD-CHAIN FATTY ACIDS ON THE BEHAVIOURAL MATURATION OF MICE

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(*Received 24 November 1980; revision received 21 October 1981*)

Abstract—Five groups of female mice were fed from day 14 of pregnancy and throughout lactation on synthetic diets differing only in the lipid component, which constituted 10% of the diet and was derived from (a) the lipid fraction extracted from yeast grown on *n*-alkanes, (b) commercial soya lecithin, (c) fatty acids isolated from yeast lipids (odd-chain fatty acids), (d) lipids extracted from yeast grown on glucose or (e) margarine (8%), corn oil (1%) and the unsaponifiable fraction (1%) from yeast grown on *n*-alkanes. A control diet (9% margarine and 1% corn oil) was fed to a sixth group of pregnant mice and to the offspring of all the groups from weaning to 60 days after birth. A battery of behavioural tests was performed on the offspring of the six groups between days 1 and 16 after birth (reflex activities), between days 2 and 21 (locomotor activity) and at 60 days of age (avoidance learning). An acceleration of postnatal behavioural maturation was evident in the pups of animals fed lipids from yeast grown on *n*-alkanes and of those fed soya lecithin, indicating the involvement of phospholipids and odd-chain fatty acids in the aspects of development studied.

INTRODUCTION

Toxicological evaluation of new food sources presents great difficulties because the administration of large doses leads to side effects due to nutritional imbalance.

Yeast biomasses containing more than 10% of lipids of unusual composition—75% phospholipids with over 50% odd-chain fatty acids (Bernardini, Boniforti, Merli & Mosuri, 1975)—can be assessed for safety by isolating the lipid fraction from the biomass itself, as suggested by Friedman, Glaser, Brown & Pariser (1971). Using this method, behavioural abnormalities were observed in rats (Gozzo, Oliverio, Salvati *et al.* 1978), in spite of the fact that odd-chain fatty acids seemed to be metabolized and incorporated normally into various tissues (Bernardini, Salvati, Serlupi-Crescenzi *et al.* 1978b,c) without affecting functional capacity (Bernardini, Salvati & Serlupi-Crescenzi, 1978a).

It has been suggested that behavioural changes may serve as a sensitive indicator of subtle functional disorders provoked by toxic agents, particularly in the developing organism (Barlow & Sullivan, 1975; Spyker, 1975). Studies on the importance of dietary lipids for prenatal growth and postnatal behaviour are relatively few but they indicate that lipids play an important role in brain and behavioural development. A number of findings have indicated that the administration of a lipid-free diet or of a diet deficient in essential fatty acids during pregnancy modifies the lipid biochemistry of the brain and affects postnatal motor reflexes, electrocorticographic activity and learning ability in the offspring (Galli, Messeri, Oliverio & Paoletti, 1975). In particular, a prenatal deficiency of essential fatty acids delays neural and behavioural maturation. It has also been shown that when synthetic diets containing a lipid fraction very rich in phospholipids and odd-chain fatty acids are administered during pregnancy and lactation, behavioural development is accelerated and brain biochemistry and morphology are modified in the offspring (Gozzo *et al.* 1978; Gozzo, Salvati & Di Felice, 1981). The latter data, obtained using the lipid fraction of yeasts grown on *n*-alkanes, are interesting both from a nutritional and from a neurobiological point of view, since they indicate that it is possible by diet-

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Abbreviations: Diets—C/A = Candida on alkanes, C/G = Candida on glucose, OFA = odd-chain fatty acids, SL = soya lecithin and USP = unsaponifiable.

Table 1. *Composition of the lipid fraction of different diets administered to pregnant and lactating mice*

Diet	Concentration of lipid components (%*)			
	Unsaponifiable	Phospholipids	Fatty acids	Odd-chain fatty acids
Candida on *n*-alkanes (C/A)	7	72	64	56
Candida on glucose (C/G)	6	27	74	Trace
Soya lecithin (SL)	1	58	73	Trace
Odd-chain fatty acids (OFA)	—	—	95	56

*Percentage of total lipids, except for odd-chain fatty acids (expressed as the percentage of total fatty acids).

ary means to interfere with brain development and to accelerate some patterns of its maturation. It was not possible in the earlier experiments to establish whether the effects on brain and behavioural maturation could be ascribed to the presence of odd-chain fatty acids or of phospholipids in the synthetic diet. The study described here was designed to clarify this point.

EXPERIMENTAL

Test diets. The five test diets and the control diet were isocalorific (at 4·35 Cal/g) and all contained casein (at a level of 21%), rice starch (53%), sucrose (10%), salt mix (4%), vitamin mix (1%) and cod-liver oil (1%). The control diet contained, in addition, 10% of lipids made up of margarine (9% of the diet) and corn oil (1%). In the test diets, this 10% lipid component was replaced by (a) a lipid fraction extracted from yeast (Toprina®) grown on *n*-alkanes (Candida on alkanes [C/A] diet), (b) commercial soya lecithin, a mixture of phospholipids (SL diet), (c) fatty acids isolated from Toprina lipids (odd-chain fatty acids [OFA] diet), (d) lipids extracted from yeast grown on glucose (Candida on glucose [C/G] diet) and (e) margarine (8%), corn oil (1%) and an unsaponifiable fraction (1%) extracted from Toprina (unsaponifiable [USP] diet). The composition of the lipid fraction of Toprina and the fatty acid components were reported by Gozzo *et al.* (1978).

Table 1 summarizes the composition of the lipid fractions of the first four test diets. Like the original Toprina fraction, the fatty-acid preparation contained a high proportion of odd-chain fatty acids, but not in the form of phospholipids. The lipid extract from glucose-grown Candida had a lower content of phospholipids than did the Toprina extract and virtually no odd-chain fatty acids. Soya lecithin was a mixture of phospholipids (58%) and glycerides containing about 18% lecithin.

Animals and treatment. One hundred female CD mice from Charles River Laboratories (Como, Italy) were mated at 60 days of age, and from day 14 of gestation, calculated from the presence of a vaginal plug, roughly equal numbers of dams received one of each of the six diets described above. Since under our experimental conditions about 75% of these mice have a gestation period of 21 days from the presence of a vaginal plug, the 60 litters (ten for each diet) used in this study were taken from those delivered on day 21. Each litter was reduced or enlarged to eight pups at birth, a size that ensured an adequate supply of milk. Feeding of the appropriate diet to the dams was continued throughout lactation. When 21 days old, the pups were sexed and weaned on to the control diet, which was fed to all 480 pups until they were 60 days old.

Behavioural tests. Pups from each dietary group were subjected at different ages to a series of tests of reflex responses, locomotor activity and avoidance learning. Reflex responses and aspects of external physical development were determined daily from day 1 to day 16 of age using a battery of behavioural tests reported elsewhere in detail (Oliverio, Castellano & Messeri, 1972; Oliverio, Castellano & Puglisi-Allegra, 1975) and described briefly here in the order in which they were carried out:

(i) Rooting reflex (does the pup raise its head in response to a gentle touch of the snout with a cotten swab?)
(ii) Cliff aversion (does the pup withdraw from the edge of a flat surface when its snout and forepaws are placed over the cliff?)
(iii) Hair growth (are the body and tail covered with hair?)
(iv) Ear opening (are the auditory canals fully open?)
(v) Righting (is the pup capable of returning rapidly to its feet when placed on its back?)
(vi) Forelimb placing (does the pup place its forepaw on cardboard that is stroked against the dorsal face of the paw?)
(vii) Forelimb grasping (does the pup grasp strongly the barrel of a 16-gauge needle touched to the palm of each forepaw?)
(viii) Bar holding (can the pup hold itself on a wooden stick (2·0 mm in diameter) for 5 sec?)
(ix) Vibrissae placing reflex (does the pup place its forepaw onto a cotton swab that is stroked across its vibrissae?)
(x) Eye opening (are the eyelids completely open?)
(xi) Auditory startle response (does the pup show a whole-body startle response when a loud snap of the fingers occurs less than 10 cm away?)

For each of these tests the score for each mouse was the age (in days) at which the finding approximated to the mature response or condition.

Locomotor activity was measured daily between day 2 and day 21 of age, in a glass cylinder (10 cm in diameter) resting on a selective Activity Meter (from Columbus Instruments, Columbus, OH), the counts for 10-min periods being recorded.

On reaching 60 days of age the pups were given a

Table 2. *Maternal body weights and pup weights for groups of mice fed diets differing only in lipid composition*

Diet	Mean body weight (g)	
	Dams after parturition	Day-old pups
Control	30·0 ± 1·5	1·45 ± 0·11
Candida on *n*-alkanes (C/A)	31·2 ± 2·1	1·51 ± 0·18
Soya lecithin (SL)	29·8 ± 1·7	1·47 ± 0·21
Odd-chain fatty acids (OFA)	32·1 ± 2·0	1·38 ± 0·20
Candida on glucose (C/G)	30·6 ± 1·4	1·49 ± 0·19
Unsaponifiable (USP)	29·7 ± 1·5	1·50 ± 0·21

Values are means ± SEM for groups of ten dams or 80 pups. There were no significant differences (ANOVA) between groups in either the maternal weights ($F = 0{\cdot}591$, $P > 0{\cdot}05$) or the pup weights ($F = 0{\cdot}410$, $P > 0{\cdot}05$).

100-trial avoidance-learning session on each of five consecutive days. This testing was carried out in an automated bank of eight shuttle-boxes, using the methods already described (Oliverio *et al.* 1972). The score for each mouse was the mean of the number of avoidances in the 100 trials in each of the five sessions.

RESULTS

The feeding of the different diets had no significant effect on the body weights of the dams (Table 2) or of the pups soon after birth (Table 2) or at 2, 4, 8, 12, 21 or 60 days of age (df = 5/63, $F = 0{\cdot}781$, $P > 0{\cdot}05$). Table 3 shows that many of the reflexes studied reached maturity more quickly in the test groups than in the controls. In the pups of the C/A group, forelimb placing and grasping, bar holding, vibrassae placing and startle all appeared at an earlier age than in control mice. Several of these reflex activities also appeared at an earlier age in the pups of the SL and OFA groups, while the performance of pups born to mothers fed the C/G diet was similar to that of the control group and the USP group showed significant retardation in some respects.

Table 3 also indicates that young mice from the C/A, SL and OFA groups showed a decrease in exploratory activity up to day 8, compared with the controls (ANOVA: df = 1/67, $F = 16{\cdot}3$, $28{\cdot}1$ and $17{\cdot}5$ respectively, $P < 0{\cdot}01$). In contrast, mice fed the USP diet were more active than control mice (df = 1/67, $F = 3{\cdot}87$, $P < 0{\cdot}05$). Finally the levels of avoidance response (Table 3) were significantly higher in the SL group (df = 1/67, $F = 13{\cdot}2$, $P < 0{\cdot}01$) and lower in the USP group (df = 1/75, $F = 16{\cdot}5$, $P < 0{\cdot}01$) than in the controls, while those of the other three groups were similar to the control performance.

DISCUSSION

In general, these findings establish that changes in the composition of the lipid fraction of a diet may affect brain maturation and development and that these changes can occur in the absence of any significant changes in food consumption or growth rate. Data reported here and other unpublished results indicate that the test diets consumed in this study did not affect the weight of pregnant or lactating rats.

The diet containing an unsaponified steroid fraction (USP diet) adversely affected behavioural maturation, as shown by the late appearance of forelimb grasping and vibrissae placing, with an accompanying delay in hair growth. It is possible that these effects were due to a steroid hormone-like factor, since steroids may delay cerebral development (Balázs & Cotterrell, 1972).

Feeding odd-chain fatty acids (the OFA diet) resulted in an earlier appearance of forelimb grasping, bar holding, vibrissae placing and to some extent startle, effects seen to a more marked degree in pups from the group fed the whole lipid fraction of yeast grown on *n*-alkanes (the C/A diet) and previously seen in rats on that diet (Gozzo *et al.* 1978). These findings were probably due to the chemical form in which the fatty acids were administered. The SL diet also induced similar effects but to a lesser degree. No effect was induced by the CG diet, lipids of which did not contain odd-chain fatty acids and included only a low proportion of phospholipids.

All the diets found to influence reflex development affected the same reflexes. Although several of these, such as forelimb grasping and bar holding, are tests of similar functions and are related to myelinization processes, the effects are much more marked in some cases, such as forelimb grasping, than in others. Preliminary data suggest that a myelin subfraction isolated, by centrifugation and a discontinuous sucrose gradient, from myelin taken from pups born to dams fed Toprina-derived lipids differs in biochemical composition and form from a similar subfraction from a group on a standard diet (Conti, Salvati, Serlupi-Crescenzi *et al.* 1980). Therefore the changes in reflex activities may reflect changes in myelinization processes. Gozzo *et al.* (1981) also showed that ingestion of lipids derived from Toprina affected the morphology of the brain cortex in the rat.

In connection with the results of the locomotor tests, in which young mice from the C/A, SL and OFA groups showed a decrease and those from the USP group an increase in exploratory activity, it is interesting to note that undernourished animals tend to be more active than controls (Blizard & Randt, 1974).

The data on avoidance learning in Table 3 show that the effects on behavioural maturation were long-lasting, particularly in the SL and USP groups, which

Table 3. *Studies of development of pups born to mice fed diets of varied lipid composition during pregnancy and lactation*

Parameter or time‡	Diet....	Score† for groups fed control or test diet					
		Control	Candida on *n*-alkanes	Soya lecithin	Odd-chain fatty acids	Candida on glucose	Unsaponifiable
Physical and reflex development§‖							
Ear opening		14·0 ± 1·0	14·0 ± 1·5	14·5 ± 1·0	14·0 ± 1·0	14·0 ± 1·5	14·5 ± 1·0
Righting		4·0 ± 0·5	3·0 ± 0·5	3·5 ± 0·5	4·0 ± 1·0	4·5 ± 1·0	4·0 ± 0·5
Rooting		1·0 ± 0	1·0 ± 0	1·0 ± 0	1·0 ± 0	1·0 ± 0	1·0 ± 0
Cliff aversion		3·5 ± 0·5	3·5 ± 0·5	3·5 ± 0	3·5 ± 0·5	4·0 ± 0·5	3·0 ± 1·0
Hair growth		11·0 ± 1·0	10·5 ± 1·0	11·0 ± 0·5	10·5 ± 0·5	10·5 ± 1·0	13·5 ± 0·5*
Forelimb placing		5·0 ± 1·0	4·0 ± 0·5	4·5 ± 1·0	4·5 ± 0·5	5·0 ± 1·0	5·5 ± 0·5
Forelimb grasping		11·5 ± 1·0	6·0 ± 0·5**	9·0 ± 0·5*	8·0 ± 0·5**	11·0 ± 0·5	14·5 ± 1·0*
Bar holding		14·0 ± 0·5	10·0 ± 0·5**	12·5 ± 0·5	11·5 ± 0·5*	14·0 ± 1·0	14·5 ± 1·5
Vibrissae placing		8·0 ± 0·5	4·5 ± 1·0**	6·6 ± 0·5*	6·0 ± 0·5*	8·0 ± 0·5	10·5 ± 0·5*
Startle		14·0 ± 1·0	11·5 ± 0·5*	12·5 ± 0·5	12·0 ± 0·5*	14·0 ± 1·0	14·5 + 1·0
Locomotor activity‖							
Day 2		20·6 ± 3·2	17·9 ± 2·6**	18·1 ± 2·9**	18·0 ± 3·0**	21·4 ± 2·8	23·7 ± 2·9
4		55·2 ± 6·4	23·9 ± 8·5**	29·1 ± 3·4**	27·5 ± 5·1**	54·8 ± 7·2	75·8 ± 6·2
8		65·2 ± 7·1	39·5 ± 7·2**	45·3 ± 8·0**	40·7 ± 6·8**	60·9 ± 8·6	88·9 ± 5·9
12		120·8 ± 11·2	125·8 ± 6·2	115·9 ± 12·0	118·6 ± 9·9	128·1 ± 10·9	134·6 ± 10·5
21		150·9 ± 13·9	158·6 ± 18·1	146·6 ± 14·0	156·2 ± 17·0	158·9 ± 19·1	158·7 ± 14·2
Avoidance learning‖							
Session 1		8·0 ± 1·0	6·9 ± 1·5	18·5 ± 1·2**	8·7 ± 2·1	6·6 ± 2·3	5·0 ± 1·0**
2		20·2 ± 2·8	19·7 ± 2·1	38·2 ± 2·6**	21·8 ± 3·1	22·6 ± 2·7	8·1 ± 2·7**
3		38·6 ± 3·1	34·5 ± 4·1	55·5 ± 4·8**	37·1 ± 2·8	40·1 ± 4·5	15·3 ± 1·9**
4		45·9 ± 6·2	42·8 ± 7·1	63·8 ± 2·7**	46·2 ± 4·7	48·9 ± 7·0	25·6 ± 2·7**
5		64·6 ± 5·7	66·1 ± 5·8	75·9 ± 7·0**	65·2 ± 6·1	66·8 ± 6·0	40·0 ± 3·1**

†Means ± SEM for groups of 16 mice derived from four litters. Each reflex test was carried out twice.

‡Locomotor activity was studied on days 2–21 after birth and avoidance learning on five consecutive days from day 60 after birth.

§Scores, determined in duplicate tests, are ages (in days) at which an approximately mature response was achieved. Those marked with asterisks differ significantly from the control values: $*P < 0.5$; $**P < 0.01$.

‖Statistical analyses (ANOVA) were carried out on a pup and litter basis by subtracting the 'within litters' variability from the residual sum of squares for all the studies.

showed higher and lower levels of avoidance behaviour, respectively, compared with controls.

Since the diets were administered during both pregnancy and lactation it is not possible to indicate which developmental period was affected. Previous experiments have shown that odd-chain fatty acids are able to pass through the placental barrier (Bernardini *et al.* 1975) and into the milk of lactating rats (Bernardini *et al.* 1978a). Therefore it is possible that odd-chain fatty acids act on brain development during both the gestational and early postnatal period.

Our results support the suggestions of Barlow & Sullivan (1975) that the monitoring of behavioural development may be a useful tool in food toxicology, allowing for a broadening of the toxicological profile. Moreover, while new synthetic diets with unorthodox lipid compositions must be carefully screened for possible effects on brain development, the use of a series of diets varying in lipid composition may be an important research tool for studies of developmental neurobiology and psychobiology.

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DIFFERENTIAL EFFECTS OF FOLIC ACID ON WATER CONTENT, PROTEIN AND MICROSOMAL 5'-PHOSPHODIESTERASE ACTIVITY OF THE RAT KIDNEY

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(*Received 14 August 1981*)

Abstract—The effects of folic acid administration on the weight, protein, water content and microsomal 5'-phosphodiesterase of the rat kidney were determined, to elucidate the mechanisms contributing to the renal enlargement produced by this agent. Folic acid administered ip in single doses of 100–250 mg/kg caused dose-related increases in kidney weight, water and protein content within 24 hr. Time-course studies indicated that 250 mg folic acid/kg given ip produced a progressive elevation in renal water content from 2 to 72 hr. Smaller increases in whole-kidney protein were recorded 8, 24 and 72 hr after folic acid treatment. However, a biphasic response of microsomal 5'-phosphodiesterase was produced, inhibition at 16 hr being followed by stimulation (to 140% of control) at 72 hr. *In vitro* studies indicated that folic acid inhibits 5'-phosphodiesterase competitively, and the early inhibition of 5'-phosphodiesterase *in vitro* appears to be due to a direct effect of folic acid on the enzyme.

INTRODUCTION

Compensatory renal enlargement follows unilateral nephrectomy, urethral ligation or administration of large doses of folic acid. In each of these situations, renal enlargement is associated with increases in renal DNA, protein, RNA and water content. However, the time course and magnitude of these changes differ markedly and the mechanisms involved are poorly understood (Johnson & Roman, 1966; Malt, 1969; Threlfall, Taylor & Buck, 1967).

It has been suggested that accumulation of renal RNA may be attributable to decreased RNA degradation since RNA stabilization occurs during compensatory renal enlargement (Melvin, Kumar & Malt, 1976). A reduction in the activity of the RNA degradative enzymes, alkaline microsomal RNAase and 5'-phosphodiesterase, is associated with drug-induced hepatic microsomal RNA and protein accumulation (Louis-Ferdinand & Fuller, 1970 & 1972; Morais, Miron, Daoust & de Lamirande, 1972). Since folic acid has been shown to inhibit RNA degradation competitively *in vitro* (Sawada, Kanesaka & Irie, 1977), the present investigation was conducted to determine the effect of folic acid treatment on renal microsomal 5'-phosphodiesterase activity and to relate these effects to the renal enlargement produced by this agent.

EXPERIMENTAL

Chemicals. Chemicals used were of analytical-reagent grade or the equivalent. Folic acid and *p*-nitrophenylthymidine 5'-monophosphate were obtained from Sigma Chemical Co., St. Louis, MO.

Animals and treatment. Male Sprague–Dawley rats (weighing 250–300 g) were used throughout these experiments. They were maintained in an air-conditioned room on a 12-hr light/dark cycle, and were given food and water *ad lib.* Folic acid dissolved in 0·3 M-sodium bicarbonate was administered ip in single doses of 100–250 mg/kg and the rats were killed by decapitation after various intervals. The vehicle was given to control groups. Dosing was timed so that all animals were killed between 09.00 and 11.00 hr.

Tissue processing and analyses. Livers and kidneys were removed when the animals were killed, rinsed with ice-cold 0·25 M-sucrose, blotted dry, weighed and minced and, using a teflon-glass homogenizer at 1000 rpm for 1 min, were homogenized with 4·0 ml 0·25 M-sucrose/g tissue. Homogenates were centrifuged in a Sorvall (RC-2B) refrigerated centrifuge for 30 min at 9000 **g**. The 9000-**g** supernatants were centrifuged for 1 hr in a Beckman (L2-65B) ultracentrifuge at 105,000 **g**. The 105,000-**g** pellets were rinsed twice, resuspended in 0·25 M-sucrose and stored in a Revco freezer at −65°C until used.

Phosphodiesterase I (5'-phosphodiesterase) activity was determined by a modification of the spectrophotometric procedure described by Morais *et al.* (1972). Incubations were carried out in a Dubnoff incubator for 15 min at 37°C. Each incubation flask contained, in a total volume of 2·5 ml, 0·1 ml 0·30% Triton X100, 0·5 ml 0·1 M-Tris-HCl buffer (pH 9·0), 0·5 μmol $MgCl_2$, 0·5 mg *p*-nitrophenylthymidine 5'-monophosphate and a microsomal or homogenate suspension equivalent to 1 mg tissue. Incubations were terminated by the addition of 2·0 ml 0·1 M-NaOH. Absorbances of samples were measured at 400 nm using a Gilford 240 spectrophotometer and were compared with those of standard *p*-nitrophenol solutions. Activity was expressed as μmol *p*-nitrophenol produced/min/kidney or /mg protein.

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Protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The water content and dry weight of the kidneys were determined by drying to constant weight at 100–110°C. For histological examination, kidneys were fixed in 10% buffered formalin and coded haematoxylin–eosin-stained sections were evaluated by a veterinary pathologist. Differences between means were determined by ANOVA and Dunnett's multiple range test (Dunnett, 1955).

RESULTS

Table 1 shows the 24-hr effect of various folic acid doses on kidney weight, kidney/body weight ratios and kidney water content. Administration of folic acid produced a dose-related increase in kidney weight within 24 hr. The highest dose (250 mg/kg) produced a significant increase of 45% in kidney weight. Kidney/body weight ratios were significantly elevated by 19, 35 and 55% of the control value by a dose of 100, 150 and 250 mg/kg respectively. These increases were paralleled by a concomitant dose-related increase in renal water content of 6, 28 and 52% of the control figure.

Figure 1 shows the effect of folic acid (250 mg/kg, ip) on rat-kidney size, 2 and 24 hr after treatment. Within 2 hr renal enlargement was discernible and a band of yellow crystalline material was evident in the medulla. By 24 hr this band was easily discernible and renal enlargement was pronounced.

To evaluate the time course of folic acid effects, a single dose (250 mg/kg, ip) was administered and the animals were killed at various intervals thereafter. Table 2 presents the time course of folic acid's effect on kidney dry and wet weights and kidney/body weight ratios. This dose of folic acid significantly increased kidney/body weight ratios and kidney wet weight at each time interval. Significant elevations in kidney dry weight were recorded 8, 24 and 72 hr after the folic acid injection, but these were not large enough to account for the observed increases in kidney weight.

Histological examination of kidneys 16 and 72 hr after ip injection of 250 mg folic acid/kg revealed no glomerular alterations. Both cortical and medullary tubules contained an acidic material of varying consistency. The tubular epithelium adjacent to this material was slightly swollen, but remained intact at most sites. Minimal nephrocalcinosis and a few pyknotic nuclei and mitotic figures were observed at 16 hr. Similar but slightly more extensive changes were observed at 72 hr.

Figure 2 compares the time course of the effects of folic acid treatment (250 mg/kg, ip) on renal water, protein content and microsomal 5′-phosphodiesterase activity. Significant increases in renal water content were observed at each examination time. Renal water content was increased by 37% of the control value within 2 hr while maximum elevation of renal water content (by 75% of control) was produced 24 hr after folic acid treatment. Significant elevations in whole-kidney protein were produced at 8 hr (by 19%), 24 hr (35%) and 72 hr (24%).

Renal microsomal 5′-phosphodiesterase showed a biphasic response to folic acid treatment. Within 2 hr of treatment, this 5′-phosphodiesterase activity decreased to 87% of control activity and a significant reduction was observed at 16 hr (to 81% of control), while at 24 hr no difference was detectable (97% of control) and at 72 hr there was a significant (35%) increase.

Folic acid was also added to incubations to determine its *in vitro* effect on the 5′-phosphodiesterase of hepatic and renal microsomal fractions. Results presented in Fig. 3 show that folic acid inhibits the microsomal 5′-phosphodiesterase of both tissues *in vitro*, 50% inhibition being produced by 2×10^{-5} and 4×10^{-5} M-folic acid in liver and renal preparations, respectively. Analysis of the double-reciprocal plots presented in Fig. 4 indicated that folic acid inhibited renal microsomal 5′-phosphodiesterase in a competitive manner and that the inhibitor constant (Ki) for folic acid was $0{\cdot}51 \times 10^{-5}$ M.

DISCUSSION

The administration of single large doses of folic acid produces renal enlargement. However the characteristics of folic acid-induced renal hypertrophy appear to differ from those observed following unilateral nephrectomy (Threlfall, Taylor & Buck, 1966 & 1967). Taylor, Threlfall & Buck (1968) reported that iv administration of folic acid doses similar to those used in the present investigation produced renal water retention which was associated with a 1·7-fold increase in kidney weight 4 days after the treatment.

Table 1. *Effect of ip administration of a single dose of 100–250 mg folic acid/kg on kidney weight and water content in the rat*

Folic acid dose (mg/kg)	No. of rats/group†	Kidney weight‡ (g)	Relative kidney weight§ (g/100 g body weight)	Water content (g H_2O/kidney)
0	5	1·07 ± 0·04 (100)	0·717 ± 0·018 (100)	0·837 ± 0·038 (100)
100	4	1·13 ± 0·04 (105)	0·855 ± 0·012* (119)	0·885 ± 0·033 (106)
150	5	1·33 ± 0·11 (125)	0·967 ± 0·080* (134)	1·069 ± 0·095 (128)
250	5	1·55 ± 0·06* (145)	1·114 ± 0·042* (156)	1·271 ± 0·056* (152)

†The animals were killed 24 hr after treatment.
‡Single kidney.
§Both kidneys.
Values are expressed as means ± SEM for the numbers of rats indicated and, in brackets, as a percentage of the control value. Those marked with an asterisk differ significantly ($P \leqslant 0{\cdot}05$) from the control value.

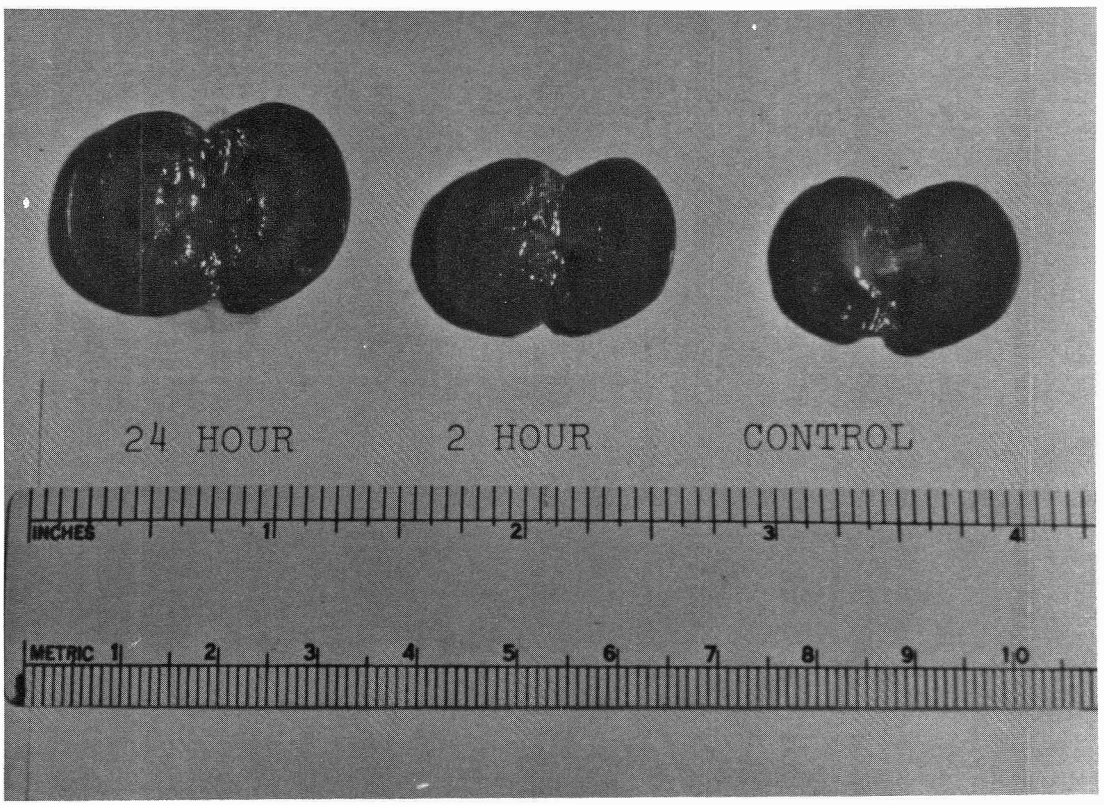

Fig. 1. Kidneys from a control rat and from rats killed 2 and 24 hr after receiving an ip injection of 250 mg folic acid/kg, showing the differences in size and in the amount of (yellow) deposit in the region of the renal medulla.

Table 2. *Time-dependent effects of a single ip dose of 250 mg folic acid/kg on kidney wet and dry weights and kidney/body weight ratio in the rat*

Time after treatment (hr)	No. of rats/group	Kidney weight (g)†		Relative kidney weight‡ (g/100 g body weight)
		Wet	Dry	
(Control)§	10	1·11 ± 0·02 (100)	0·253 ± 0·006 (100)	0·711 ± 0·013 (100)
2	4	1·43 ± 0·05* (128)	0·273 ± 0·011 (108)	0·902 ± 0·038* (126)
8	5	1·55 ± 0·03* (139)	0·277 ± 0·006* (109)	1·023 ± 0·013* (143)
16	4	1·47 ± 0·03* (132)	0·267 ± 0·009 (106)	0·991 ± 0·017* (140)
24	5	1·80 ± 0·06* (163)	0·306 ± 0·008* (121)	1·204 ± 0·043* (170)
72	4	1·78 ± 0·14* (161)	0·329 ± 0·015* (130)	1·199 ± 0·125* (169)

†Single kidney.
‡Both kidneys.
§Two control rats were killed at each examination time, after treatment with the vehicle (0·3 M-sodium bicarbonate).
Values are expressed as means ± SEM for the numbers of rats indicated and, in brackets, as percentage of the control value. Those marked with an asterisk differ significantly ($P \leq 0·05$) from the control value.

Time-course studies in the present investigation indicated that a 1·4-fold increase in renal water content occurs within 2 hr of folic acid treatment. Furthermore, a 1·7-fold increase in water content at 24 hr persists through 72 hr. Results of the present study show that the primary contribution to increased renal weight during the early phase of folic acid-induced renal enlargement is water accumulation.

Renal folic acid deposition may also contribute to the observed early increases in kidney weight. Histological examination revealed minimal necrotic changes up to 72 hr following ip administration of 250 mg folic acid/kg. The findings were consistent with those reported by Baserga, Thatcher & Marzi (1968) following administration of the same dose to mice. In rats, Huguenin, Birbaumer, Brunner *et al.* (1978) observed folic acid casts in the proximal tubule, ascending limb of Henle's loop and collecting ducts within 15 min of iv administration of 250 mg folic acid/kg. This is compatible with the observations of Taylor *et al.* (1968) who reported that a significant fraction of injected folic acid is deposited in rat kidneys. These authors have suggested that the renohypertrophic effect of folic acid is due to an increased "functional load" on remaining tubules resulting from partial tubular blockage caused by folic acid precipitation in the renal tubules.

Although renal water content was highest 24 and 72 hr after folic acid treatment, a 1·3–1·4-fold elevation in kidney protein was also observed at these times. Furthermore, 5'-phosphodiesterase activity was elevated 1·4-fold by 72 hr. The present findings are compatible with those of Threlfall & Taylor (1969) who have shown that renal protein synthesis increases five-fold between 16 and 24 hr after folic acid treatment (250 mg/kg).

5'-Phosphodiesterases of the rat kidney and liver are recovered primarily in microsomal fractions where they participate in the sequential degradation of nucleic acids (Razzell, 1961). Folic acid inhibition of this enzyme is not tissue-specific since both renal and hepatic microsomal 5'-phosphodiesterases were inhibited by folic acid *in vitro*. The renal 5'-phosphodiesterase responds to folic acid administration in a biphasic manner, a 20% reduction in its activity at 16 hr being followed by a 1·4-fold elevation at 72 hr. Phosphodiesterase inhibition may contribute to the early rapid accumulation of renal RNA following folic acid administration when compared to nephrectomy-induced hypertrophy (Threlfall *et al.* 1967).

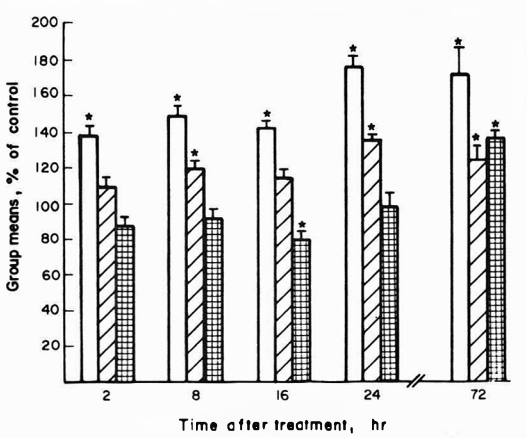

Fig. 2. Time course of the effects of an ip dose of 250 mg folic acid/kg on water (□) and protein (▨) contents and 5'-phosphodiesterase activity (▦) of the rat kidney. The control values (means ± SEM) were 0·849 ± 0·021 g H_2O/kidney, 155·56 ± 4·33 mg protein/kidney and 21·62 ± 0·98 μmol *p*-nitrophenol formed/kidney/min. Asterisks indicate significant ($P \leq 0·05$) values.

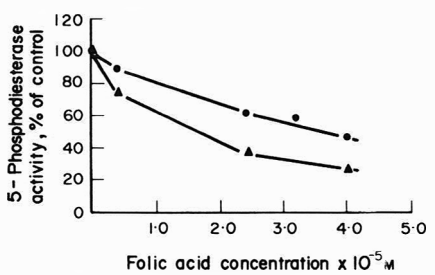

Fig. 3. Comparison of the *in vitro* effects of folic acid on renal (●) and hepatic (▲) 5'-phosphodiesterase studied in microsomal preparations from the pooled kidneys or livers of four male rats. Control values were 0·644 and 0·149 μmol *p*-nitrophenol formed/mg protein/min for kidney and liver, respectively.

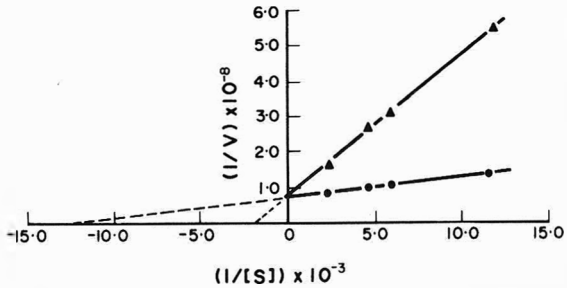

Fig. 4. Double-reciprocal plot showing the mechanism of inhibition of 5′-phosphodiesterase by folic acid ($3{\cdot}6 \times 10^{-5}$ M). Each point (folic acid, ▲; control, ●) represents the value obtained with microsomes of pooled kidneys from four male rats. Velocities (V) are in mol/litre/min, substrate concentrations ([S]) in mol/litre. $Km = 7{\cdot}7 \times 10^{-5}$ M and $Ki = 0{\cdot}51 \times 10^{-5}$ M.

The initial decrease in 5′-phosphodiesterase activity may be attributable to direct effects of folic acid. Although renal folic acid concentration was not determined in the present study, folic acid is known to be deposited rapidly in the kidneys of rats (Huguenin *et al.* 1978) and renal tubular cells accumulate reabsorbed folic acid (Goresky, Watanabe & Johns, 1963). Beyond 24 hr, folic acid dilution by the additional renal water and *de novo* protein synthesis may be responsible for the observed elevation in 5′-phosphodiesterase activity.

In summary, the results of this investigation suggest that folic acid treatment produces an early and persistent dose-related increase in the water content of the kidney as well as a biphasic alteration in renal 5′-phosphodiesterase activity. The early reduction of 5′-phosphodiesterase activity may be partially attributable to direct effects of folic acid, while its subsequent elevation appears to be associated with compensatory renal enlargement.

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N-NITROSO-*N*-METHYLDODECYLAMINE AND *N*-NITROSO-*N*-METHYLTETRADECYLAMINE IN HAIR-CARE PRODUCTS*

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Abstract—*N*-Nitroso-*N*-methyldodecylamine and *N*-nitroso-*N*-methyltetradecylamine, which cause urinary bladder tumours in experimental animals, were detected in several hair-care products formulated with *N*,*N*-dimethyldodecylamine oxide. Quantitative determinations were made using a gas–liquid chromatograph interfaced with a thermal energy analyser and using [1-^{14}C]*N*-nitroso-*N*-methyldodecylamine as an internal standard. The presence of the two nitrosamines was confirmed by high-pressure liquid chromatography with a thermal energy analyser as detector, by photolysis of samples and by combined gas chromatography–mass spectrometry. To test the reproducibility of the method, a single shampoo was selected for replicate analysis and was found to contain 90 ± 8 ppb *N*-nitroso-*N*-methyldodecylamine and 37 ± 11 ppb *N*-nitroso-*N*-methyltetradecylamine. Levels of *N*-nitroso-*N*-methyldodecylamine in other hair-care products ranged from 11 to 873 ppb and those of *N*-nitroso-*N*-methyltetradecylamine from 8 to 254 ppb.

INTRODUCTION

Certain cosmetics products are contaminated with *N*-nitrosodiethanolamine, an animal carcinogen which is formed by nitrosation of the widely used ingredients diethanolamine and triethanolamine (Fan, Goff, Song *et al.* 1977). Cosmetics contain numerous other amines, amine oxides and ammonium salts which are potential precursors to carcinogenic nitrosamines. Therefore, nitrosamines other than *N*-nitrosodiethanolamine might also be present in cosmetics. This could be important because the carcinogenicities of nitrosamines depend to a great extent on the nature of their substituent groups (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Magee, Montesano & Preussmann, 1976). *N*,*N*-Dimethyldodecylamine oxide (lauramine oxide) is one of the ingredients which we considered as a likely precursor to nitrosamines in cosmetics. In the present study, we have demonstrated that several hair-care products formulated with lauramine oxide contain *N*-nitroso-*N*-methyldodecylamine ($CH_3 \cdot N(NO) \cdot [CH_2]_{11} \cdot CH_3$) and *N*-nitroso-*N*-methyltetradecylamine ($CH_3 \cdot N(NO) \cdot [CH_2]_{13} \cdot CH_3$). Both of these nitrosamines are carcinogenic in experimental animals (Althoff & Lijinsky, 1977; Cardy & Lijinsky, 1980; Ketkar, Althoff & Lijinsky, 1981; Lijinsky, Saavedra & Reuber, 1981; Lijinsky & Taylor, 1975).

EXPERIMENTAL

Apparatus. Quantitative analyses for *N*-nitrosamines were accomplished by GLC using a Hewlett-Packard Model 700 gas chromatograph† and a Model 543 Thermal Energy Analyzer (TEA; Thermo Electron Corp., Waltham, MA) connected by means of a modification described elsewhere (Brunnemann & Hoffmann, 1981). The GLC column was a 12 ft × 2 mm ID glass tube packed with 10% UCW-982 on Gas-Chrom Q 80/100. The oven was operated isothermally at 210°C. For high-pressure liquid chromatography (HPLC), a Model 45 solvent delivery system (Waters Associates, Milford, MA), a Model 7105 syringe loading sample injector (Rheodyne, Berkeley, CA), a LiChrosorb Si-60 analytical HPLC column (25 cm × 4·6 mm ID) (E. Merck Labs, Elmsford, NY) and a Model 502 LC Thermal Energy Analyzer (Thermo Electron Corp.) were connected in sequence.

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

†Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the Food and Drug Administration and does not imply its approval to the exclusion of other products that may also be suitable.

Abbreviations: NMDDA = *N*-Nitroso-*N*-methyldodecylamine; NMTDA = *N*-Nitroso-*N*-methyltetradecylamine; GLC = Gas–liquid chromatography; TEA = Thermal Energy Analyzer; GC–MS = Gas chromatography–mass spectroscopy; TLC = Thin-layer chromatography.

A Hewlett-Packard Model 5710A gas chromatograph with a flame-ionization detector was used for conventional GLC. The column was a 6 ft × 2 mm ID glass tube filled with 3% OV-101 on Gas-Chrom Q 100/120. Combined GC–MS were recorded on a Hewlett-Packard Model 5982A mass spectrometer equipped with the same column. A Nuclear-Chicago Isocap 300 scintillation system (Nuclear Chicago Corp., Des Plaines, IL) was used for liquid-scintillation counting. Radiochromatography was performed using a Packard Model 7201 Radiochromatogram Scanner (Packard Instruments, Downers Grove, IL). For photolysis experiments, the ultraviolet light source was a Model UVSL-25 Mineralight Lamp (UltraViolet Products, Inc., San Gabriel, CA) operated at 366 nm.

Materials. Consumer hair-care products were purchased in retail stores or obtained from regional distributors. Gas chromatographic packings were from Applied Science Laboratories, Inc. (State College, PA). Silica-gel (Baker-analysed, 40–140 mesh; J. T. Baker Chemical Co., Phillipsburg, NJ) and *N*-methyldodecylamine (Pfaltz and Bauer, Inc., Stamford, CT) were used without further purification. Thin-layer chromatography (TLC) plates were 0·25 mm silica-gel 60 F-254 (E. Merck, Darmstadt, West Germany). Liquid-scintillation counting cocktail was prepared from ScintiPrep 2 (Fisher Scientific Company, Fairlawn, NJ). Ethanol-free chloroform for HPLC was obtained from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). All other solvents were of spectral purity.

N-*Nitroso*-N-*methyldodecylamine.* An aqueous solution of *N*-methyldodecylamine (2·0 g, 0·01 mol) and sodium nitrite (0·9 g, 0·013 mol) was stirred at pH 3–4 for 17 hr at room temperature. The product was extracted with chloroform and purified by elution from a silica-gel column with chloroform–methanol (15:1, v/v). Further purification was accomplished by TLC on silica-gel with elution by chloroform (R_F = 0·26). The mass spectrum of NMDDA (Fig. 3) was identical to that previously reported (Rainey, Christie & Lijinsky, 1978). Purity of greater than 99% was established by GLC. The oven was operated isothermally at 180°C with a helium flow rate of 60 ml/min.

N-*Nitroso*-N-*methyltetradecylamine. N*-Methyltetradecylamine (1·1 g, 0·005 mol; ICN, Plainview, NY) was stirred for 20 hr with sodium nitrite (3·4 g, 0·05 mol) in dilute acetic acid. The nitrosated product was extracted with chloroform and purified by column chromatography on silica-gel with elution by hexane and hexane–methylene chloride (1:1, v/v). Further purification by TLC as for NMDDA yielded NMTDA (R_F = 0·26) with a mass spectrum (Fig. 4) identical to that reported previously (Lijinksy *et al.* 1981). GLC at 150°C established purity greater than 99%.

[*1-*^{14}*C*]N-*nitroso*-N-*methyldodecylamine.* [1-^{14}C]-Lauric acid (250 μCi; 32 μCi/μmol; Amersham, Arlington Heights, IL) was dissolved in 2 ml methylene chloride and sealed under nitrogen in a round-bottom flask equipped with a magnetic stirrer. Oxalyl chloride (0·5 ml) was added, and the mixture was stirred for 24 hr. The solvent and unreacted oxalyl chloride were removed under a stream of dry nitrogen, and the residue was redissolved in 2 ml methylene chloride. Methylamine gas (Matheson, East Rutherford, NJ) was bubbled into the solution until the pH reached 12, and the solvent was again removed with a gentle stream of nitrogen. The residue was dissolved in 2 ml freshly distilled tetrahydrofuran and reduced with lithium aluminium hydride (2 mg) by heating under reflux for 10 hr. The mixture was cautiously diluted with distilled water and extracted with methylene chloride. The combined extracts were concentrated, dissolved in 3 ml distilled water at pH 3 and stirred for 14 hr with sodium nitrite (3 mg). The product was extracted with methylene chloride and purified by silica-gel TLC with elution by chloroform–methanol (20:1, v/v), yielding 18·5 μCi [1-^{14}C]NMDDA (7·4% from [1-^{14}C]lauric acid). Radiochemical purity of greater than 99% was established by radiochromatography using the TLC system above.

Quantitative analysis for NMDDA and NMTDA in hair-care products by GLC–TEA. A mixture of 10 g hair-care product, 50 mg ascorbic acid and [1-^{14}C]NMDDA (5·5 × 10^3 cpm, 22 ng) as internal standard was stirred for 1 min in a 250 ml Erlenmeyer flask. Diethyl ether (100 ml) was added and stirring was continued for 15 min. Anhydrous sodium sulphate (40 g) was added, and stirring was continued for 15 min more. The mixture was filtered, rinsed with 50 ml diethyl ether and concentrated by rotary evaporation at 35°C after the addition of 2 ml *n*-butanol to reduce foaming. The residue was applied to a silica-gel column (20 g) packed in hexane and eluted with chloroform (260 ml). The eluate was collected as one fraction, evaporated to dryness under reduced pressure at 35°C and redissolved in either 1·0 or 2·0 ml chloroform. A 2–10 μl aliquot of this solution was analysed by GLC–TEA.

Analysis by HPLC–TEA. The sample preparation was identical to that described above. A 50–100 μl aliquot of the final chloroform solution was analysed by HPLC–TEA, operating the instrument isocratically using chloroform–cyclohexane–ethanol (700:300:1, by vol.) at a flow rate of 1 ml/min.

Photolysis of samples. Each sample was analysed by GLC–TEA and HPLC–TEA before and after photolysis. For these studies, 30–100 μl of the chloroform solution was transferred into a thin-walled, melting point capillary tube, which was placed 6 cm from the ultraviolet light source and exposed for 2 hr.

Analysis by GC–MS. A 90 g sample of a commercial product (Hot-oil treatment, lot 1, Table 2) mixed with ascorbic acid (500 mg) and [1-^{14}C]NMDDA (5·5 × 10^3 cpm, 22 ng) was stirred with diethyl ether (300 ml) for 30 min. Anhydrous sodium sulphate (150 g) was added, and stirring was continued for 20 min. The mixture was filtered, concentrated and loaded onto a silica-gel column (125 g). The column was eluted first with 400 ml hexane and then with hexane–methylene chloride (1:1, v/v). After the hexane eluate was collected, fractions of 20 ml were collected. The radioactive fractions were combined, concentrated, applied to silica-gel TLC plates and developed with chloroform *v.* NMDDA standard. The band corresponding to NMDDA was removed and extracted with methanol. The methanol was evaporated and the residue was redissolved in chloroform and analysed by GLC–MS. The gas-chromatograph

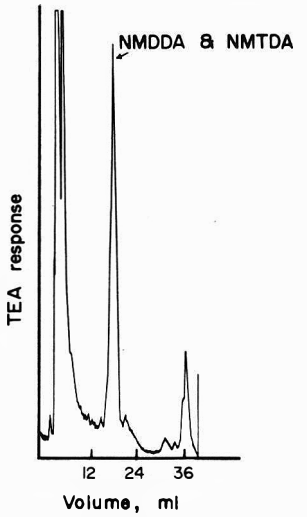

Fig. 1. HPLC–TEA chromatogram of NMDDA and NMTDA in a hair-care product.

oven was temperature programmed from 120 to 250°C at 1°/min, and the flow rate of helium was 50 ml/min.

RESULTS

Analyses of hair-care products were carried out using synthetic [1-^{14}C]NMDDA as a tracer and internal standard. Products were stirred with ether, and the ether extracts were dried and passed through silica-gel prior to analysis by HPLC–TEA and GLC–TEA. A typical HPLC–TEA chromatogram of a product containing NMDDA and NMTDA is shown in Fig. 1. The indicated peak co-eluted with both NMDDA and NMTDA standards, which were not separated under these conditions. Other TEA-positive peaks were also present in every sample. Analysis by GLC–TEA gave a typical chromatogram as shown in Fig. 2. Under these conditions, NMDDA and NMTDA were separated and both peaks co-eluted with reference standards. When samples were exposed to ultraviolet light and reanalysed by HPLC–TEA

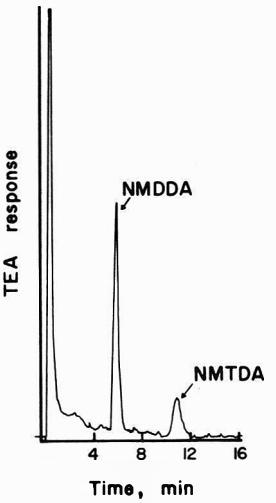

Fig. 2. GLC–TEA chromatogram of NMDDA and NMTDA in a hair-care product.

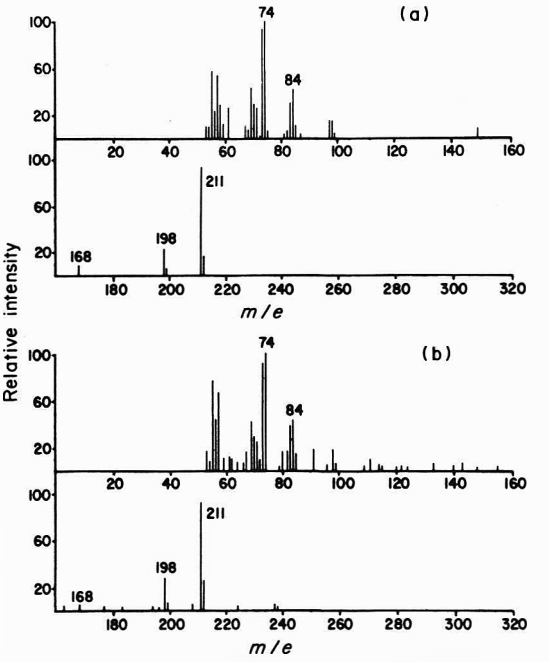

Fig. 3. Mass spectra of (a) reference NMDDA and (b) NMDDA isolated from a hair-care product.

and GLC–TEA, the peaks corresponding to NMDDA and NMTDA decreased or disappeared, as expected for nitrosamines (Krull, Goff, Hoffman & Fine, 1979). To confirm the presence of these nitrosamines, one sample was processed on a larger scale and analysed by GLC–MS. Peaks corresponding in retention time to NMDDA and NMTDA were observed, and their mass spectra were identical to those of reference standards as shown in Figs 3 and 4. The two major high-mass peaks in both spectra are due to loss of fragments with *m/e* 17 and *m/e* 30 from the molecular ions, as is typical for *N*-nitrosamines.

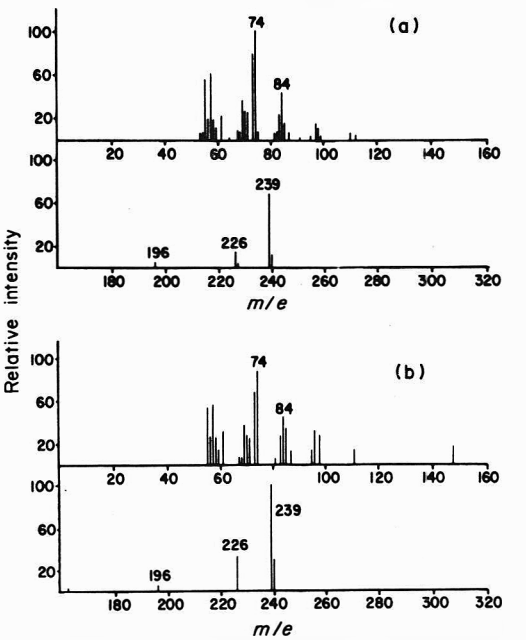

Fig. 4. Mass spectra of (a) reference NMTDA and (b) NMTDA isolated from a hair-care product.

Table 1. *Reproducibility of analyses for NMDDA and NMTDA in shampoo A, Lot 1*

Analysis	Recovery of [1-^{14}C]NMDDA (%)	NMDDA (ppb)	NMTDA (ppb)
1	80	84	30
2	92	80	27
3	80	92	31
4	83	100	51
5	86	94	47
Mean	84	90	37
Standard deviation	5	8	11

Quantitative analyses were performed by GLC–TEA. The results of five analyses of shampoo A, lot 1, are presented in Table 1. Model studies showed that recoveries of NMDDA and NMTDA were comparable. Therefore, values for NMDDA and NMTDA were corrected for recoveries of [1-^{14}C]NMDDA. Recoveries ranged from 63 to 98%. We considered the possibility that NMDDA and NMTDA could have been formed artefactually despite the use of ascorbic acid in the extraction procedure. Therefore, we performed an additional experiment in which we added 1 mg $NaNO_2$ and 1 mg *N*-methyldodecylamine to shampoo A, lot 1. Analysis revealed 97 ppb NMDDA, indicating that artefact formation was not a significant problem. Levels of NMDDA and NMTDA in several hair-care products are summarized in Table 2.

DISCUSSION

The results of this study demonstrate that NMDDA and NMTDA are contaminants in several hair-care products formulated with lauramine oxide. The presence of NMDDA and NMTDA was confirmed by GC–MS analysis of one sample. However, all samples for which values are reported in Table 2 gave positive GLC–TEA and HPLC–TEA responses for both compounds. The GLC–TEA results were particularly informative since all positive samples gave traces similar to that shown in Fig. 2, and the peaks decreased in intensity or disappeared upon photolysis.

Table 2. *NMDDA and NMTDA in hair-care products*

Product	Concentration (ppb)* NMDDA	NMTDA
Shampoo A, lot 1	90	37
Shampoo A, lot 2	92	34
Shampoo A, lot 3	49	22
Shampoo A, lot 4	203	76
Shampoo B	17	8
Shampoo C	16	10
Hair conditioner A	11	9
Hair rinse A, lot 1	46	22
Hair rinse A, lot 2	ND	ND
Hair rinse A, lot 3	11	ND
Hair rinse A, lot 4	83	25
Hot oil treatment, lot 1	604	218
Hot oil treatment, lot 2	873	254

ND = Not detected
*[1-^{14}C]NDOMA was used as internal standard.

The presence in these products of both NMDDA and NMTDA suggests that they have similar precursors which are probably the C_{12} or C_{14} dimethyl amine oxides or the corresponding tertiary or secondary amines (Lijinsky, Keefer, Conrad & Van de Bogart, 1972). Related alkyl amines or amine oxides which are present in these products could also be converted to the corresponding nitrosamines. Other nitrosamines were not characterized in the present study, but most of the samples gave HPLC–TEA peaks in addition to the one observed in each case for NMDDA and NMTDA.

The results of this study suggest that various nitrosamines may be present in cosmetics products that are formulated with ingredients derived from amines. The most direct approach to controlling this potential problem is the identification and elimination of nitrosating agents in cosmetics or the use of inhibitors of nitrosation. However, inhibitors will have to be effective against the formation of both hydrophilic nitrosamines such as *N*-nitrosodiethanolamine and lipophilic nitrosamines such as NMDDA and NMTDA.

NMDDA induces primarily bladder tumours in rats, Syrian golden hamsters and European hamsters and liver tumours in guinea-pigs (Althoff & Lijinsky, 1977; Cardy & Lijinsky, 1980; Ketkar *et al.* 1981; Lijinsky & Taylor, 1975). NMTDA as well as *N*-nitroso-*N*-methyloctylamine and *N*-nitroso-*N*-methyldecylamine, also induces urinary bladder tumours in rats (Lijinsky *et al.* 1981). Extensive dose-response data are not available for NMDDA and NMTDA, but it has been suggested that they are considerably less potent carcinogens than are the methylalkyl nitrosamines with alkyl chain lengths of 3 to 5 (Lijinsky *et al.* 1981). However, NMDDA is apparently more carcinogenic in rats and Syrian golden hamsters than is *N*-nitrosodiethanolamine (Althoff & Lijinsky, 1977; Habs, Preussmann & Schmähl, 1981; Lijinsky & Taylor, 1975; A. Rivenson, S. S. Hecht & D. Hoffmann, unpublished data 1981). Further studies are clearly necessary to evaluate human risk from exposure to NMDDA and NMTDA. In the meantime, it would be prudent to eliminate NMDDA and NMTDA from cosmetics.

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MUTAGENICITY OF COMMERCIAL HAIR DYES IN *SALMONELLA TYPHIMURIUM* TA98

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Abstract—Commercial permanent hair-dye formulations containing *p*-phenylenediamine, resorcinol and aminophenols were incubated with hydrogen peroxide and then tested for their ability to induce reverse mutations in *Salmonella typhimurium* TA98. Approximately half of the formulations (12 out of 25) gave positive results. The activity varied widely in degree and was observed only in the presence of an S-9 microsomal fraction from Aroclor-induced male rats. Five of the 12 positive formulations and one negative dye were administered topically to male rats; with one exception the urines of animals treated with the mutagenic hair dyes gave positive results in the presence of the S-9 mix.

INTRODUCTION

Permanent hair-dye formulations consist of a mixture of aromatic amines, aromatic nitro derivatives and phenols, which are mixed with H_2O_2 just before use. Some of these ingredients have been shown to be mutagenic in bacteria (Ames, Kammen & Yamasaki, 1975; Nishioka, 1976), in yeast (Mayer & Goin, 1980) and/or in Drosophila (Blijleven, 1977), to induce chromosomal damage in cultured Chinese hamster cells (Kirkland & Venitt, 1976) and/or to be carcinogenic in rodents (Ito, Hiasa, Konishi & Marugami, 1969; National Cancer Institute, 1978; Reznik & Ward, 1979). Consequently some governments have banned the use of genotoxic ingredients or imposed warning labelling of hair-dye packages. Italy acted just after the first report of mutagenicity in *Salmonella typhimurium* (Ames *et al.* 1975) with a decree (Ministerial Decree, 18 June 1976) which banned, from 1 January 1978, the following nine chemicals widely used as primaries and couplers in permanent hair dyes: 2,4-diaminoanisole, 4-nitro-*o*-phenylenediamine, 2-nitro-*p*-phenylenediamine, 2,5-diaminoanisole, 2-amino-5-nitrophenol, *m*-phenylenediamine, *o*-phenylenediamine, 2-amino-4-nitrophenol and 2,5-diaminotoluene. Subsequently 2,4-diaminotoluene, carcinogenic in rats (Ito *et al.* 1969), was also banned (Ministerial Decree, 7 March 1979).

So far, none of the ingredients of the oxidative hair dyes now used in Italy has given evidence of mutagenicity; these formulations usually include *p*-phenylenediamine (*p*-PD), widely used as the primary dyestuff, with resorcinol and aminophenols as couplers. It is possible, however, that mutagenic agents may be present as impurities in the *p*-PD used or may be formed during the oxidation with H_2O_2. *p*-PD for example may be oxidized to a highly reactive trimer, Bandrowski's base (Corbett, 1971). Furthermore, chemicals that are not themselves mutagenic may react to generate mutagenic products, as was recently demonstrated in the conjugation of *p*-PD with resorcinol (Crebelli, Conti, Carere & Zito, 1981b).

We have therefore studied, in *S. typhimurium* TA98, the mutagenic activity of hair dyes used in Italy. The tests were carried out after a brief incubation with H_2O_2 to simulate normal conditions of use. Some attempt was also made to assess both the percutaneous absorption of the mutagenic formulations during the oxidation reaction and their urinary excretion.

EXPERIMENTAL

Test material. The following 25 product lines of ten different companies were tested: Recital A bis, B, H, S, W and Z and Preference 4 and 9.1 from l'Oreal Italiana, Saipo s.p.a., Turin; Dickson Color 4c, 6c and 8c from Dickson Service, Milan; Crystal Color 7 Garnier from Saipo s.p.a., Turin; Socolor Plus 9c from Soc. Cosmetici, Turin; Gelcolor 2n and 23r and Olicolor 18r from La Cramer S.p.A., Perugia; Akkord Color 306/o from Wella Corp., Englewood, NJ, USA; Magis 1c from Prodotti Tricosmetici, Bologna; Nature 3, 4, 6, 7r and 308 from Intercosmo, Bologna; Tricol 3c from Tricol, Milan; Faipa 2c from Faipa, Pomezia.

Mutagenicity assay. The culture of *S. typhimurium* TA98 was kindly supplied by Professor B. N. Ames, University of Berkeley, CA, USA, and the selection of induced *his*$^+$ revertants was performed according to the procedure described by Ames, McCann & Yamasaki (1975). Liver microsomal (S-9) fractions were obtained by the procedure of Ames *et al.* (1975) from male Sprague–Dawley rats weighing about 250 g and pretreated with Aroclor 1254. The hair dyes were mixed with 3% H_2O_2 (1:1, v/v) and incubated for 30 min at 37°C before testing. Dyes that gave a posi-

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tive result in this assay were also tested without the H_2O_2 pre-incubation (with and without the S-9 mix). Glusulase, a mixture of sulphatase and β-glucuronidase from Endo Laboratories (Garden City, NY, USA) was added to the urine-concentrate assays, to provide about 1000 U β-glucuronidase and 400 U sulphatase per plate. Every experiment was repeated at least twice and all chemicals were tested in a range of concentrations up to the highest non-toxic dose. 2-Aminoanthracene (1 μg/plate) was always included as a positive control to check enzyme activities and strain sensitivity, and samples and S-9 preparations were routinely checked for sterility.

Preparation of urine concentrates. Male Sprague–Dawley rats (body weight *c.* 250 g) were treated as described by Shah (1977) but without the dorsal shaving. Briefly 3 g hair dye was mixed with 3 ml 3% H_2O_2, applied for 30 min to the dorsal skin of rats and afterwards removed by shampooing. Care was taken to exclude the possibility of ingestion of any of the test material. Urine samples were collected in metabolism cages by means of the device previously described (Crebelli *et al.* 1981b). After the collection period (usually 72 hr), the XAD-2 resin (Serva Heidelberg, FRG) was washed in the cold with 100 vols water to reduce the small amount of urinary histidine present. The acetone eluate (10 + 5 ml acetone, from E. Merck, Darmstadt, FRG) was concentrated under reduced pressure and lyophilized. Dimethylsulphoxide (DMSO) (Carlo Erba S.p.A., Milan) was used to dissolve the acetone residues immediately before use.

RESULTS

For every hair-dye formulation tested, similar results were obtained in each of the replicate experiments. Table 1 reports the data from one experiment in each case. With about half of the dyes (13 out of 25) the numbers of *his*$^+$ revertants consistently recorded were not more than twice the number in the control assays and these dyes were therefore judged to be negative ($h \leqslant 1$ in Fig. 1). The other 12 dyes all

Table 1. *Mutagenicity of commercial hair dyes in* Salmonella typhimurium *TA98*

Identity of dye	Dye concn (mg/plate)	No. of *his*$^+$ revertants/plate* With H_2O_2 and S-9	No. of *his*$^+$ revertants/plate* Without H_2O_2, with S-9	Dye concn (mg/plate)	No. of *his*$^+$ revertants/plate Without H_2O_2 and S-9
Recital A bis	0	45 ± 5			
	12·5	38 ± 3			
	25	53 ± 5			
	50	58 ± 14			
Recital B	0	42 ± 1			
	12·5	30 ± 2			
	25	28 ± 5			
	50	45 ± 4			
Recital H	0	38 ± 3			
	5	39 ± 3			
	12·5	30 ± 8			
	25	8 ± 1			
Recital S	0	45 ± 5			
	12·5	28 ± 5			
	25	35 ± 6			
	50	47 ± 13			
Recital W	0	41 ± 9			
	2·5	53 ± 13			
	5	35 ± 8			
	7·5	25 ± 7			
Recital Z	0	41 ± 9			
	2·5	53 ± 10			
	5	41 ± 8			
	7·5	26 ± 5			
Preference 4	0	49 ± 4	44 ± 3	0	48 ± 4
	12·5	110 ± 13	115 ± 12	5	32 ± 3
	25	234 ± 50	86 ± 10	12·5	18 ± 2
	50	269 ± 42	toxic	25	14 ± 5
Preference 9.1	0	36 ± 2	45 ± 5	0	21 ± 4
	12·5	50 ± 6	32 ± 2	5	21 ± 3
	25	60 ± 4	32 ± 9	12·5	21 ± 2
	50	95 ± 7	26 ± 1	25	14 ± 4
Dickson Color 4c	0	31 ± 5	38 ± 2	0	31 ± 6
	12·5	92 ± 9	89 ± 11	12·5	33 ± 8
	25	94 ± 9	92 ± 1	25	24 ± 5
	50	70 ± 13	79 ± 18	50	27 ± 2
Dickson Color 6c	0	40 ± 12	45 ± 5	0	31 ± 4
	5	61 ± 4	59 ± 3	5	33 ± 7
	12·5	69 ± 12	77 ± 5	12·5	55 ± 3
	25	101 ± 5	105 ± 5	25	46 ± 2

[*contd*]

Table 1 (*continued*)

Identity of dye	Dye concn (mg/plate)	No. of *his*$^+$ revertants/plate* With H_2O_2 and S-9	No. of *his*$^+$ revertants/plate* Without H_2O_2 with S-9	Dye concn (mg/plate)	No. of *his*$^+$ revertants/plate Without H_2O_2 and S-9
Dickson Color 8c	0	42 ± 1	36 ± 7	0	19 ± 5
	12·5	69 ± 7	68 ± 10	5	25 ± 5
	25	94 ± 11	89 ± 9	12·5	19 ± 3
	50	88 ± 3	98 ± 12	25	17 ± 2
Crystal Color 7 Garnier	0	40 ± 2			
	2·5	38 ± 4			
	5	42 ± 12			
	12·5	20 ± 8			
Socolor Plus 9c	0	49 ± 6			
	12·5	46 ± 10			
	25	56 ± 4			
	50	34 ± 2			
Gelcolor 2n	0	30 ± 2	34 ± 9	0	21 ± 2
	1·25	63 ± 0	59 ± 1	1·25	12 ± 1
	2·5	78 ± 3	114 ± 9	2·5	17 ± 1
	5	100 ± 14	104 ± 13	5	20 ± 4
Gelcolor 23r	0	39 ± 3			
	2·5	37 ± 5			
	5	24 ± 6			
	12·5	16 ± 3			
Olicolor 18r	0	28 ± 6			
	0·5	41 ± 3			
	1	35 ± 0			
	2·5	37 ± 7			
Akkord Color 306/o	0	49 ± 4			
	5	60 ± 9			
	12·5	53 ± 2			
	25	73 ± 10			
Magis 1c	0	44 ± 3	44 ± 3	0	25 ± 3
	2·5	169 ± 25	123 ± 13	2·5	17 ± 5
	5	226 ± 18	106 ± 20	5	13 ± 1
	12·5	193 ± 7	34 ± 11	12·5	16 ± 4
Nature 3	0	35 ± 4	36 ± 4	0	22 ± 0
	0·5	58 ± 8	43 ± 9	0·5	23 ± 4
	1·25	126 ± 15	50 ± 13	1·25	26 ± 6
	5	295 ± 20	61 ± 14	5	17 ± 5
Nature 4	0	40 ± 1	45 ± 5	0	21 ± 4
	0·5	55 ± 7	53 ± 11	0·5	29 ± 3
	1·25	101 ± 4	57 ± 2	1·25	29 ± 5
	5	138 ± 3	44 ± 1	5	toxic
Nature 6	0	34 ± 9	35 ± 4	0	22 ± 0
	1·25	68 ± 3	42 ± 4	1·25	18 ± 3
	2·5	82 ± 8	38 ± 6	2·5	20 ± 3
	5	119 ± 27	36 ± 5	5	19 ± 3
Nature 7r	0	36 ± 2			
	2·5	33 ± 4			
	5	34 ± 1			
	7·5	23 ± 11			
Nature 308	0	37 ± 4			
	0·5	37 ± 4			
	1	40 ± 1			
	2·5	34 ± 4			
Tricol 3c	0	43 ± 4	28 ± 6	0	13 ± 1
	5	251 ± 17	255 ± 56	5	16 ± 4
	12·5	314 ± 23	330 ± 35	12·5	22 ± 2
	25	320 ± 34	431 ± 56	25	26 ± 3
Faipa 2c	0	40 ± 4	40 ± 4	0	15 ± 0
	12·5	607 ± 33	590 ± 117	5	12 ± 3
	25	905 ± 16	1016 ± 119	12·5	16 ± 6
	50	1205 ± 138	1201 ± 94	25	26 ± 3
2-Aminoanthracene†			807 ± 99		

*Expressed for each test dye as the mean ± 1 SD for three plates from one experiment.
†Positive control used at a concentration of 1 μg/plate. The value is the mean ± 1 SD of all values recorded throughout the study.

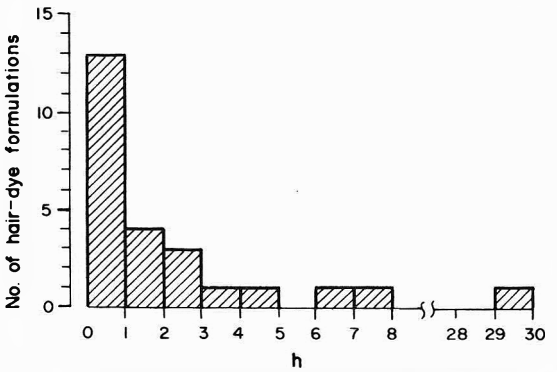

Fig. 1. Distribution of mutagenic activity in 25 commercial hair dyes tested in *Salmonella typhimurium* TA98 with metabolic (S-9) activation after incubation with H_2O_2 (from data in Table 1). Genetic activity (h) is expressed as the increase over the control value: $h = (his^+_{treated} - his^+_{control})/his^+_{control}$.

showed mutagenic activity in *S. typhimurium* TA98, but their specific activities differed widely. None showed mutagenic potential in the absence of S-9 and with some dyes the mutagenicity associated with S-9 activation was reduced (Magis 1c, Preference 4 and Nature 3) or eliminated (Nature 4 and 6 and Preference 9.1) when incubation with H_2O_2 was omitted. The distribution of levels of mutagenic activity is illustrated in Fig. 1.

Urine concentrates from untreated rats and from rats treated with one of six hair dyes showing varying degrees of mutagenicity from zero to high when tested directly against *S. typhimurium* were assayed with and without metabolic activation. The reason for using S-9 mix when testing the urines of animals treated with indirect mutagens has been discussed (Crebelli, Carere & Zito, 1981a). Briefly it is based on evidence that the more reactive metabolites of a compound are not usually found in the urine because they react with macromolecular targets in the body; frequently it is the unmetabolized fraction of the compound that is detected in the urine (Durston & Ames, 1974).

Negative results were obtained with the urines of untreated rats and of rats treated with a dye (Recital B) that was non-mutagenic in the earlier *in vitro* assay (Table 2). With one exception, the urine concentrates from animals treated topically with the mutagenic dyes showed a detectable mutagenicity when tested in the presence of S-9, although the observed activity was not quantitatively related to the *in vitro* mutagenicity. The exception was a negative result following treatment with Preference 4. None of the urine concentrates showed any mutagenicity in the absence of S-9 activation.

DISCUSSION

Several countries have recently banned the use of some well-known genotoxic agents as ingredients in hair-dye formulations. Nevertheless it appears that some of the permitted formulations still retain mutagenic potential. Various factors may determine the mutagenic properties of the particular class of hair dyes (based on *p*-PD with resorcinol and aminophenols as couplers) that were assayed in this study. In a recent investigation we demonstrated that the mutagenicity of *p*-PD depended on the purity of the sample tested (Crebelli *et al.* 1981b). This factor may vary widely in commercial products, and may thus have a marked effect on the mutagenicity of the dyes. Another possible factor in the observed mutagenicity may be the generation of mutagenic conjugates during the oxidation with H_2O_2; this could explain the oxidation-dependent activity of some dyes, such as was recently demonstrated (Crebelli *et al.* 1981b) in

Table 2. *Mutagenicity in* Salmonella typhimurium *TA98 of urine concentrates from rats treated topically with hair dyes and hydrogen peroxide*

Dye	S-9 activation	No. of *his*$^+$ revertants/plate at urine-concentrate levels (mg/plate) of: 0	0·1	0·25	0·5	1·0
None	–	31	29	31	31	30
	+	36	38	33	35	33
Recital B	–	36	38	37	24	26
	+	40	42	52	65	61
Preference 4	–	36	29	29	35	NT
	+	40	46	49	54	47
Dickson 8c	–	26	17	23	22	NT
	+	41	128**	169**	184**	NT
Tricol 3c	–	21	22	20	23	26
	+	56	87*	79*	106*	146**
Faipa 2c	–	26	26	19	25	23
	+	39	67*	96**	120**	168**
Gelcolor 2n	–	15	18	13	15	17
	+	37	51	66*	75**	83**

NT = Not tested

Values are means for three plates and those marked with asterisks differ significantly (Student's *t* test) from the control value: *$P < 0.05$; **$P < 0.01$. The standard error was never >15% of the mean.

the case of purified *p*-PD and resorcinol, neither of which was mutagenic when tested alone.

Caution is necessary when relating an ability to induce reverse mutations in this strain of bacteria to true carcinogenic potential. Nevertheless a good qualitative correlation between mutagenicity and carcinogenicity has been demonstrated in some studies, especially with aromatic amines (McCann, Choi, Yamasaki & Ames, 1975; Rinkus & Legator, 1979; Simmon, 1979). This point and our observation of percutaneous absorption of potentially mutagenic products during hair-dyeing should be given consideration as indications of potential genetic and/or carcinogenic risk. Our future goal will therefore be the identification of the mutagenic constituents of commercial hair dyes, to permit a better assessment of their genotoxic characteristics and so provide a useful basis for decisions on their removal from or replacement in commercial products.

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GENOTOXICITY OF 5-METHOXYPSORALEN AND NEAR ULTRAVIOLET LIGHT IN REPAIR-DEFICIENT STRAINS OF *ESCHERICHIA COLI* WP2

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Abstract—5-Methoxypsoralen (5-MOP) is used in cosmetic suntan preparations to stimulate the production of skin pigments. Although its isomer 8-methoxypsoralen (8-MOP) has been shown to be genotoxic in numerous biological systems, 5-MOP has not been so extensively investigated, but it has recently been reported to be mutagenic and carcinogenic. We have studied the lethal effects of 5-MOP and near ultraviolet light (NUV) on repair-deficient *Escherichia coli* strains. After treatment with 5-MOP at concentrations above 2 μg/ml in combination with UV light survival of the repair-deficient strains was considerably reduced and strain WP100 uvrA$^-$ recA$^-$ was more sensitive than strain WP2 uvrA$^-$. The effect was dependent on the time of irradiation and on the presence or absence of S-9 mix which inhibited the lethal activity of 5-MOP/NUV particularly when the NADP-generating system was included. These results support other indications that the use of 5-MOP in cosmetic preparations should be controlled.

INTRODUCTION

5-Methoxypsoralen (bergapten), a naturally occurring major ingredient of bergamot oil (Fowlks, 1959), belongs to the class of furocoumarins. Certain furocoumarin derivatives, the psoralens, have gained attention because of their toxicity to various organisms when applied in combination with light. The photosensitizing properties of psoralens, especially 8-methoxypsoralen, have lead to their use for vitiligo (Glatt, 1961) and psoriasis treatment (Scott, Pathak & Mohn, 1976; Weber, 1974). Studies of the mutagenic activity of photosensitizing agents are of interest in view of the need to assess their carcinogenic potential (Ames, Durston, Yamasaki & Lee, 1973; Pool, 1980; Purchase, Longstaff, Ashby *et al.* 1978; Sugimura, Sato, Nagao *et al.* 1976). Whereas the biological activity of 8-MOP when given in combination with near ultraviolet light has been investigated in a variety of organisms (Pool, 1981; Scott *et al.* 1976), less effort has been directed towards similar studies of its isomer 5-MOP. However, such studies are necessary, since 5-MOP finds widespread use in sunscreen and tanning preparations as a stimulant to the production of the skin pigment, melanin.

5-MOP is photoreactive and is reported to induce chromosomal aberrations in *Vicia faba* root tips (Musajo, 1955 *cited in* Scott *et al.* 1976). Recently Ashwood-Smith, Poulton, Barker & Mildenberger (1980) published a study on 5-MOP/NUV-induced lethal and mutagenic effects on *E. coli* WP2, and lethal and clastogenic effects on isolated mammalian cells. 5-MOP was more active than the monofunctional angelicin and less active than 8-MOP and psoralen in these systems.

We report here on the lethal photosensitizing properties of 5-MOP in repair-deficient *E. coli* WP2 strains. A preferential killing of the repair-deficient strains at concentrations not affecting the survival of the wild type repair-proficient strain *E. ccli* WP2 indicates the induction of lethal DNA damage. This test is a useful supplementary method for detecting genetically active compounds (Green & Muriel, 1976).

EXPERIMENTAL

Chemicals and light source. 5-MOP was obtained from Givaudan Res. Co. Ltd, Zürich, Switzerland who determined it to be analytically pure. 8-MOP of analytical grade was obtained from Fluka, Switzerland. The light source was a UV lamp F8 T5 (Vetter, Wiesloch) emitting UV light (366 nm) at an intensity of 360 mW/cm^2 at a distance of 15 cm, as determined using a digital radiometer (J-260 UV-Products, Inc. Hermic, Berlin).

Metabolizing system. S-9 was obtained from Aroclor 1254 pretreated male Sprague–Dawley rats by the procedure of Ames, McCann & Yamasaki (1975). A complete metabolizing system was provided by S-9

Abbreviations: 5-MOP = Methoxypsoralen; 8-MOP = 8-Methoxypsoralen; NUV = Near ultraviolet.

Table 1. *Survival of controls treated with DMSO only*

Experimental conditions		No. of colonies/plate of *Escherichia coli* strain		
Media	Time of irradiation (min)	WP2	WP2 uvrA	WP100 uvrA recA
Buffer	0	58 ± 4	46 ± 3	24 ± 4
		58 ± 2	58 ± 4	32 ± 3
		59 ± 2	51 ± 5	26 ± 3
		56 ± 1	58 ± 3	33 ± 3
Buffer	5	47 ± 3	48 ± 2	22 ± 4
		59 ± 1	59 ± 2	32 ± 2
Buffer	15	58 ± 2	95 ± 8	31 ± 5
		57 ± 3	74 ± 5	33 ± 3
		59 ± 3	62 ± 4	25 ± 2
		66 ± 4	66 ± 4	23 ± 2
S-9 without cofactors	15	62 ± 4	96 ± 4	37 ± 3
		63 ± 4	74 ± 4	36 ± 3
S-9 with cofactors	15	61 ± 7	98 ± 5	36 ± 4
		65 ± 3	78 ± 3	39 ± 4

Values are means ± SD for nine separate determinations in each experiment. The individual values for 2–4 independent experiments under each set of conditions are given.

mix containing per ml: 0·4 ml S-9 fraction (20 mg protein/ml), 4 μmol NADP, 4 μmol glucose-6-phosphate, 7 μmol $MgSO_4$ and 0·3 ml bacterial suspension (10^{-5} dilution from an overnight culture) in 0·05 mM phosphate buffer pH 7·4. The S-9 mix without cofactors contained 0·4 ml S-9 fraction, 0·3 ml bacterial suspension and 0·05 mM phosphate buffer in each millilitre. In both cases a final bacterial dilution of 3×10^{-6} was achieved.

Bacterial assay. Indicator strains were tryptophan auxotrophic *Escherichia coli* WP2 and the repair-deficient strains *E. coli* WP2 uvrA$^-$ and *E. coli* WP100 uvrA$^-$, recA$^-$, kindly provided by M. H. L. Green, Brighton, UK. A modification of the 'treat and plate' method proposed by Green & Muriel (1976) and described by Pool, Eisenbrand & Schmähl (1979) was used. For this, 0·5 ml of each bacterial suspension (3×10^{-6} dilution of an overnight culture) or 0·5 ml of the S-9/bacterial mixtures was incubated with 5–20 μl of the appropriate compound solution in DMSO (0·05, 1, 2, 5, 10, 12·5, 20, 25 or 50 μg compound/tube) for 5 or 15 minutes at 25°C in a shaking water bath, either with or without irradiation by NUV. After the treatment period, 10 μl of each sample were plated onto nutrient broth agar plates in triplicate. Total numbers of survivors were determined after 24 hr incubation at 37°C. Based on the absolute numbers of survivors in the untreated controls (100%) (Table 1), the percentages of survivors after treatment with 5-MOP and 8-MOP were calculated.

RESULTS

In Table 1 the mean number of surviving colonies in 10 μl solvent controls are shown. Each value is the mean of nine separate determinations, of which three groups had each contained 5, 10 and 15 μl DMSO per incubation flask, respectively. The low standard deviations indicate that DMSO added in these amounts did not affect bacterial survival. Furthermore the values from different experiments were generally well within the ranges calculated for the individual experiments, demonstrating the reproducibility of the system.

Figure 1 shows the survival of the repair deficient strains compared with the wild type strain *E. coli* WP2 in the presence of near UV light (15 min) and eight different 5-MOP concentrations. Those strains lacking uvrA excision repair are more sensitive to lethal effects in the presence of 5-MOP. Most sensitive is strain *E. coli* WP100 uvrA$^-$ recA$^-$ indicating the role of recombination repair after psoralen-induced DNA damage (Bridges, Mottershead & Knowles, 1979). The survival rate is clearly dependent on the photosensitization by UV light as is shown in Figs 2a & b. Practically no cell death is observed in the presence of 5-MOP without NUV irradiation (0 min) in either of the repair-deficient strains at the concentrations tested. In Fig. 3, the effects with 5-MOP are compared with those obtained with 8-MOP. In all three strains the higher sensitivity to 8-MOP is evident. The addition of S-9 mix with cofactors results in a distinctly reduced lethal activity of 5-MOP/NUV (15 min); S-9 mix without cofactors partially inhibits the DNA damaging effects of 5-MOP/NUV (15 min) in the repair-deficient strains (Figs 4a & b).

DISCUSSION

This study indicates that 5-MOP causes DNA

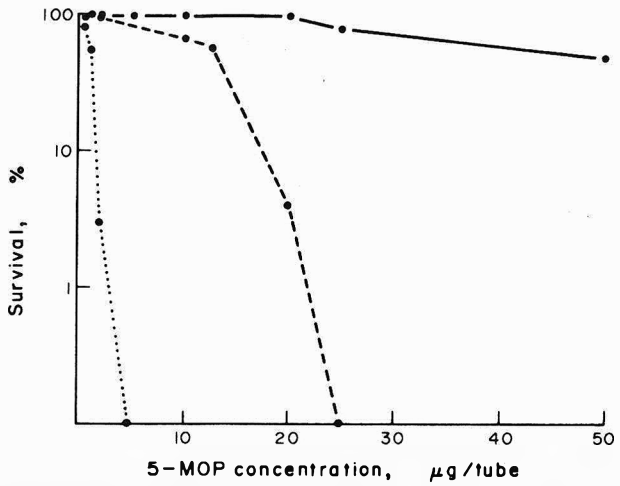

Fig. 1. Survival of *Escherichia coli* WP2 (——), *E. coli* WP2 uvrA⁻ (– – –) and *E. coli* WP100 uvrA⁻ recA⁻ (· · · · · ·) treated with various concentrations of 5-MOP, after 15 min near UV irradiation. Values are means of three separate determinations from one reproducible experiment. The extremes never differed from the means by more than 5–10%.

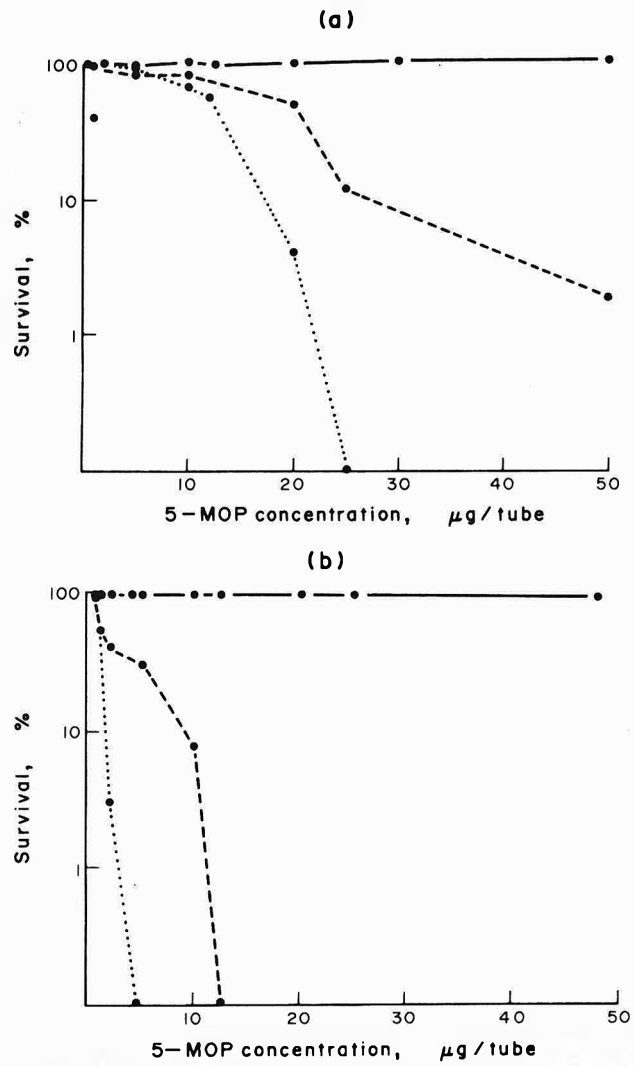

Fig. 2. Survival of (a) *Escherichia coli* WP2 uvrA⁻ and (b) *E. coli* WP100 uvrA⁻ recA⁻ after treatment with 5-MOP and incubation either for 15 min in the dark (——) or for 10 min in the the dark and 5 min irradiation with near UV (– – –) or for 15 min irradiation with near UV (· · · · ·). Values are means of three separate determinations from one reproducible experiment. The extremes never differed from the means by more than 5–10%.

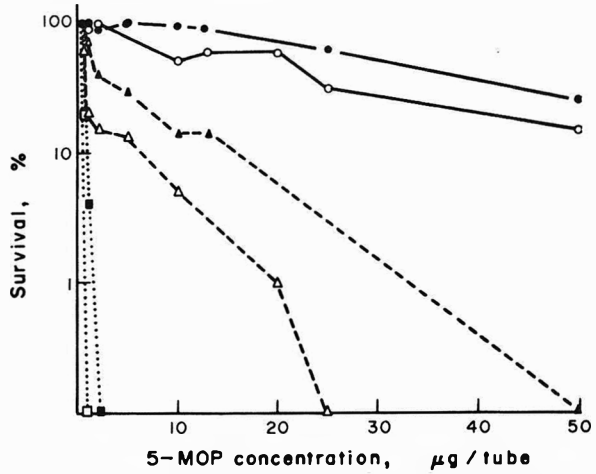

Fig. 3. Comparison of the effects of various concentrations of 5-MOP (■▲●) and 8-MOP (□△○) on the survival of *Escherichia coli* WP2 (——), *E. coli* WP2 uvrA⁻ (– – –) and *E. coli* WP100 uvrA⁻ recA⁻ (· · · · ·) after 15 min irradiation with near UV. Values are means of three separate determinations from one reproducible experiment. The extremes never differed from the means by more than 5–10%.

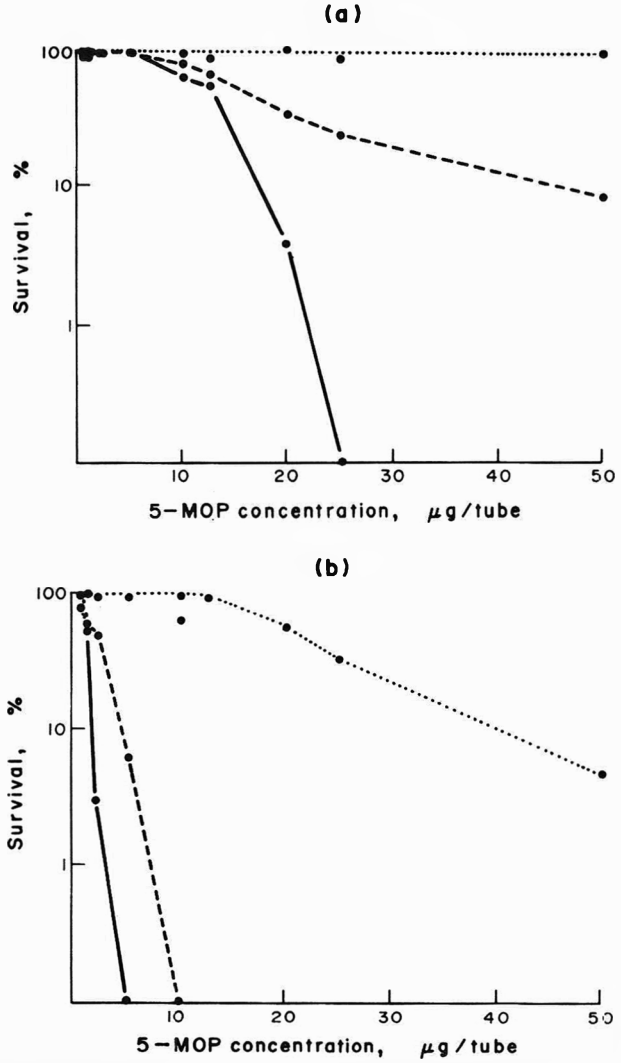

Fig. 4. Survival of (a) *Escherichia coli* WP2 uvrA⁻ and (b) *E. coli* WP100 uvrA⁻ recA⁻ after treatment with 5-MOP and 15 min near UV irradiation in the presence of buffer (——), S-9 mix without co-factors (– – –) or S-9 mix with co-factors (· · · · ·). Values are means of three separate determinations from one reproducible experiment. The extremes never differed from the means by more than 5–10%.

damage in repair-deficient *E. coli* strains after photosensitization by NUV. Its activity is weaker than that of 8-MOP. 8-MOP itself causes mono adducts and DNA crosslinks reparable by excision and recombination repair in *E. coli* (Bridges *et al.* 1979; Igali, Bridges, Ashwood-Smith & Scott, 1970). Ashwood-Smith *et al.* (1980) have demonstrated a multitude of mutagenic, clastogenic and lethal effects induced by 5-MOP/NUV. Recently Zajdela & Bisagni (1981) have demonstrated the carcinogenic activity of 5-MOP in mice after topical administration of the compound and irradiation with NUV. Again the activity was close to that of psoralen and 8-MOP, but had a longer latent period. Our study also shows that 5-MOP was deactivated by rat-liver preparation (S-9 mix). The effect of S-9 without cofactors may be attributable to the action of cytosolic enzymes, to an interaction of protein with 5-MOP or to the absorption of NUV by S-9 decreasing the irradiation intensity. The increased deactivation by the complete S-9 mix containing NADP-generating cofactors may be due to UV absorption by these cofactors and/or metabolism and deactivation of 5-MOP by cytochrome-dependent enzymes. Similar deactivation was observed for 8-MOP (Shimmer & Fischer, 1980) in a test using arginine-dependent Chlamydomonas as indicator organisms. Observations of this kind have led to the oral administration of 8-MOP (Parrish, Fitzpatrick, Tannenbaum & Pathak, 1974; Wolff, Fitzpatrick, Parrish *et al.* 1976) in psoriasis treatment. Whereas this may provide a means of diminishing the risk involved in a beneficial medical therapy, different criteria must be used in evaluating cosmetic preparations. We therefore support the proposal of previous authors (Ashwood-Smith *et al.* 1980; Zajdela & Bisagni, 1981) that the use of 5-MOP and 8-MOP in suntan preparations should be carefully evaluated and controlled.

Note added in proof—We have also recently observed bacteriophage induction by 5-MOP/NUV and 8-MOP/NUV using an assay developed by Elespuru (1981).

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PRESENCE OF BENZO[*a*]PYRENE AND OTHER POLYCYCLIC AROMATIC HYDROCARBONS IN SUNTAN OILS

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Abstract—Column and thin-layer chromatography followed by spectrophotofluorometry were used to determine the carcinogen benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons (PAH) in commercial samples of four suntan oils based on mineral and/or vegetable oils liable to contain traces of PAH. The analyses showed that all the samples contained benzo[*a*]pyrene together with three other mutagenic, co-carcinogenic or non-carcinogenic PAH (perylene, fluoranthene and benzo[*k*]fluoranthene). One sample also contained the carcinogen anthanthrene. The total PAH content of the samples varied from 88·5 to 188·7 ng/g while benzo[*a*]pyrene levels were in the 1·5–45·7 ng/g range. The results suggest that users of suntan oils may be exposed to low levels of potentially hazardous PAH; however, in comparison with many other cosmetics that are presumably based on similar oils, suntan oils are only used intermittently and for relatively short periods.

INTRODUCTION

Recently, some authors have reported the presence of carcinogens or mutagens in cosmetics or in ingredients of cosmetics (Ames, Kammen & Yamasaki, 1975; Fan, Goff, Song & Fine, 1977; Muzzall & Cook, 1979; Prival, Mitchell & Gomez, 1980; Searle, Harnden, Venitt & Gyde, 1975). In previous work we found trace amounts (ng/g) of PAH in refined petroleum products used in cosmetics and medicinal products (Monarca, Morozzi & Fagioli, 1981). Two of these PAH (B*a*P and benzo[*b*]fluoranthene) were carcinogenic and five were non-carcinogens. PAH are ubiquitous and abundant environmental pollutants and some are highly suspect as causative agents in the induction of cancer of the skin as well as of other organ systems in man (Andrews, Thibault & Lijinsky, 1978; Hoffmann, Schmeltz, Hecht & Wynder, 1978; Hoffmann & Wynder, 1977; IARC Working Group, 1973).

Fully-refined petroleum products, with a potential trace-level content of PAH, constitute ingredients of many cosmetic preparations, such as cold creams, cleansing creams, brilliantines, lipsticks, massaging oils, foundation make-up, eye shadow, baby lotions or creams and suntan oils (Balsam & Sagarin, 1972; Jellinek, 1970; Kraft, Hoch, Quisno & Newcomb, 1972; McCarthy, 1976; Poucher, 1974). In addition, some cosmetics are prepared from vegetable oils, which may also contain trace amounts of B*a*P and other PAH (Ciusa, D'Arrigo, Maini & Penna, 1968; Ciusa & Morgante, 1980; Ciusa, Nebbia, Buccelli & Volpones, 1965; Grimmer, Hildebrandt & Böhnke, 1975; Howard & Fazio, 1980; Howard, Turiochi, White & Fazio, 1966. Joe, Roseboro & Fazio, 1979; Lintas, De Matthaeis & Merli, 1979; Siegfried, 1975).

Concern about photochemical activation processes in skin carcinogenesis has led some authors to study the interaction of PAH and UV irradiation or visible light in connection with skin cancer in animals, but results have been conflicting (Cavalieri & Calvin, 1971; Forbes, Davies & Urbach, 1976; Santamaria, Giordano, Alfisi & Cascione, 1966; Stenbäck, 1975; Suskind, 1977). More recently, it has been reported that near-ultraviolet irradiation of cultured mammalian cells in the presence of B*a*P induces mutations in the absence of a metabolic activation system, suggesting that this PAH can be activated by a photo-induced process (Barnhart & Cox, 1980; Hoard, Ratliff, Bingham & Strniste, 1981).

The purpose of the present work was to apply a previously developed analytical procedure for the determination of PAH in pharmacopoeial paraffin samples (Monarca, 1980; Monarca *et al.* 1981) to commercial samples of mineral- and/or vegetable-based suntan oils, which are applied to the skin and exposed to sunlight. Particular emphasis was placed on the determination of B*a*P, the most studied and one of the most carcinogenic PAH.

EXPERIMENTAL

Materials. The suntan-oil samples studied were chosen from the commercial products most commonly used in Italy, in order to provide samples of different composition (those based exclusively on mineral oil or on vegetable oil and a mixed composition). Table 1 shows the declared composition of the base for each formulation. For each sample the contents of several containers were mixed to provide a large homogeneous volume (2–3 litres) for the setting-up of the analytical procedure and its application.

Determination of PAH. The analytical procedure was a modified version of a previously developed

Abbreviations: B*a*P = Benzo[*a*]pyrene; PAH = polycyclic aromatic hydrocarbon(s).

Table 1. *Declared composition of the base material of each suntan oil*

Suntan-oil sample	Declared base composition*	Type of oil
A	Liquid paraffin (high purity)	Mineral
B	Walnut oil	Vegetable
C	Natural and pure vegetable oils	Mixed vegetable
D	Coconut oil, cocoa butter, liquid paraffin, olive oil, almond oil, lanoline oil	Mineral and mixed vegetable

*As listed on the container label.

method (Monarca, 1980; Monarca *et al.* 1981) and consisted of the following steps: (a) PAH extraction by frontal elution chromatography; (b) two thin-layer chromatographic PAH separations; (c) PAH determination by spectrophotofluorometry. The losses occurring during this analytical procedure were determined by adding a known amount of tritiated B*a*P (0·052 μCi).

Extraction procedure. The presence of trace amounts (ng/g) of PAH in these samples required a very efficient extraction procedure. The method previously developed for paraffin samples was applied to the suntan-oil samples (method 1, as shown in Fig. 1) but gave rise to some problems, including low percentage recoveries and inadequate clean-up leading to considerable volumes of extractives. A modified method (method 2) was therefore developed, by changing the ratio of silica gel to sample (from 1:1 to 2:1, w/w) and also modifying the column elution (Fig. 2). The volume of eluting solvents was chosen by

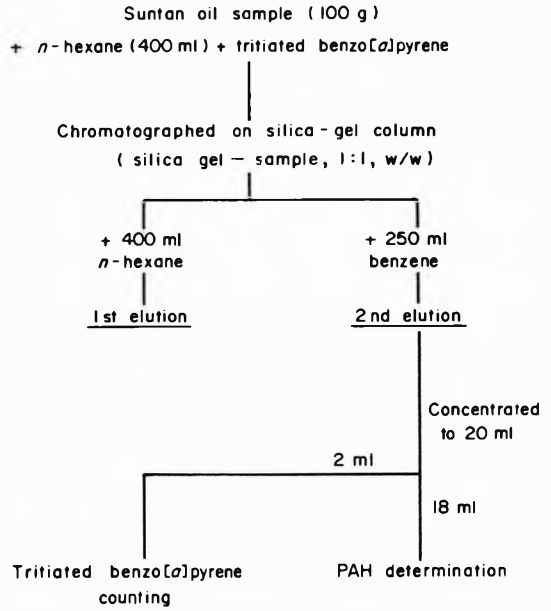

Fig. 1. Method 1 for polycyclic aromatic hydrocarbon (PAH) extraction from suntan oils by frontal elution chromatography.

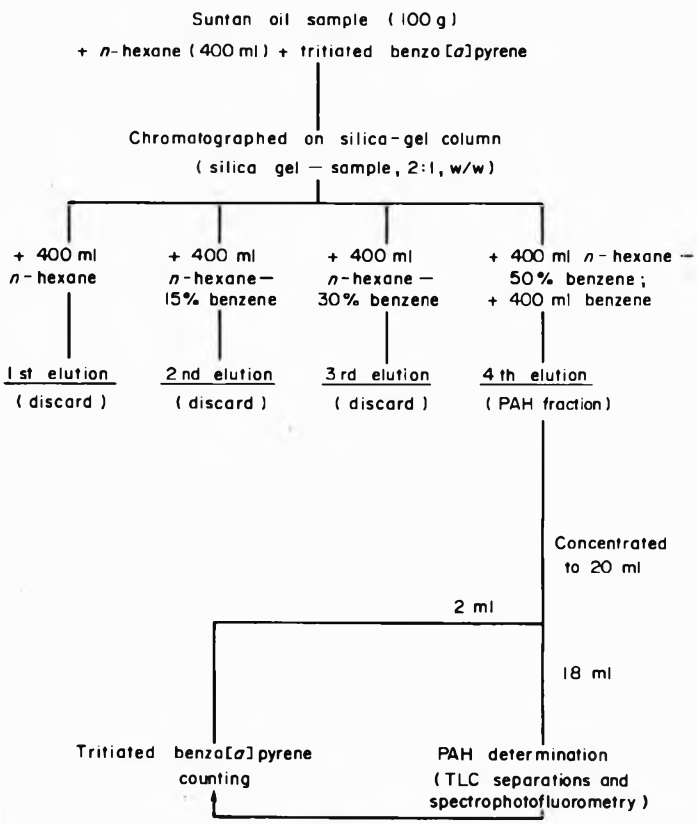

Fig. 2. Method 2 for polycyclic aromatic hydrocarbon (PAH) extraction from suntan oils by frontal elution chromatography.

performing tests in which suntan-oil samples were added to a mixture of standard PAH (4 μg of each PAH) and the elution of these compounds was followed by using UV lamps (254 and 366 nm wavelengths) and recording the excitation and emission spectra of 25-ml fractions.

Chromatographic separations of PAH. The extracts obtained by the second extraction procedure (preferred for its higher extraction and clean-up efficiency) were submitted to two sequential thin-layer chromatographic separations.

In the first, the PAH chromatographic pre-separation, the extracts were applied in lines on silica gel-precoated plates (2 mm thick, E. Merck AG, Darmstadt, FRG), and then developed with cyclohexane–benzene (1:1, v/v); a mixture of standard PAH was spotted near the extracts for marking the PAH areas using UV lamps (254 and 366 nm wavelengths). The adsorbent areas with the same R_F values as the standard PAH were scraped off, ground and extracted in small sintered glass filter funnels, first with 60 ml benzene and then with 60 ml dichloromethane (spectrograde; Carlo Erba SpA, Milan).

In the second chromatographic separation, the extracts derived from the PAH areas of the silica-gel plates were applied to 30% acetylated cellulose-precoated plates (0·10 mm thick, 30 Acetil CE; Carlo Erba SpA) in lines and the chromatograms were developed by a blend of ethanol–dichloromethane–water (20:10:1, by vol.). The single fluorescent lines were scraped off and extracted in small sintered glass filter funnels, first with 40 ml benzene and then with 40 ml ethyl ether (spectrograde; Carlo Erba SpA).

PAH determination by spectrophotofluorometry. The dried extracts of the acetylated-cellulose thin-layer chromatograms were taken up in specific volumes of spectrograde cyclohexane (Carlo Erba SpA). In these diluted solutions PAH determinations were performed by spectrophotofluorometry, using a previously described procedure (Monarca, 1980). Losses incurred during the extraction procedures were calculated from counts of the remaining tritiated B*a*P.

Table 2. *Recoveries of tritiated benzo*[a]*pyrene (BaP) from suntan oils after column chromatography (method 2) and after the entire analytical procedure*

Suntan-oil sample	Recovery of [^{3}H]B*a*P (%)	
	PAH extraction*	Full analytical procedure†
A	82·9 ± 4·8	50·3
B	78·8 ± 5·2	46·0
C	92·8 ± 6·5	59·0
D	85·4 ± 7·1	52·0

*Recovery determined after frontal elution chromatography (method 2) of the suntan oils. Values are means ± 1 SD for four determinations.

†Recovery determined on final solutions prepared by successive chromatographic separations for spectrophotofluorimetric analysis. Values are means of two determinations.

RESULTS

Extraction

The first extraction method gave good results (82·8% recovery) only for sample A (which had a mineral-oil base), the extraction efficiencies being very low for the other samples: 51·8% for B, 46·0% for C and 41·5% for D. Furthermore, residues of the extractions of samples B, C and D (containing vegetable oils) were considerable (some millilitres) and this hindered the subsequent chromatographic separations.

These problems were resolved by using the second extraction method, which gave a high percentage recovery for all the samples (78·8–92·8%; Table 2) and small volumes of extractives. The recoveries of the labelled internal standard for the whole analytical procedure (second extraction method and the PAH determination) were acceptable (46·0–59·0%) for all the samples (Table 2).

PAH determinations

The analytical procedure used demonstrated the presence of five PAH, namely B*a*P, a known carcinogen, anthanthrene, also carcinogenic, benzo[*k*]fluoranthene, fluoranthene and perylene. Of these, anthanthrene was present only in sample C but the other four PAH were present in all the samples.

The levels of PAH isolated are shown in Table 3. These values were corrected for losses as indicated by the recovery of tritiated B*a*P added at the beginning of the analytical procedure. The levels of B*a*P were submitted to two distinct corrections, one by applying the factor for the losses indicated by the recovery of tritiated B*a*P and the other by subtracting the B*a*P content added as the labelled internal standard.

The PAH levels varied in the samples examined. Maximum values for B*a*P and total PAH were found in sample C (based on mixed vegetable oils) and minimum values in sample A (with a mineral-oil base). Only the sample based exclusively on mixed vegetable oils contained anthanthrene. The total PAH levels

Table 3. *Concentrations of benzo*[a]*pyrene (BaP) and other polycyclic aromatic hydrocarbons in suntan-oil samples*

Polycyclic aromatic hydrocarbon	Concentration (ng/g)* in suntan oil:			
	A	B	C	D
Benzo[*a*]pyrene†	1·5‡	3·6‡	45·7‡	21·4‡
Anthanthrene†	ND	ND	1·2	ND
Benzo[*k*]fluoranthene	1·3	1·5	1·6	2·1
Fluoranthene	116·3	78·4	135·4	75·2
Perylene	2·0	5·0	4·8	3·4
Total PAH content	121·1	88·5	188·7	102·1

ND = Not detectable

*All values were corrected for losses in accordance with the recovery of added tritiated benzo[*a*]pyrene (Table 2) and are means of two determinations.

†Carcinogenic hydrocarbon (Andrews *et al.* 1978; Hoffmann & Wynder, 1977; IARC Working Group, 1973).

‡Values were submitted to two distinct corrections, for losses indicated by the level of recovery of tritiated B*a*P and for the amount of B*a*P added as the labelled internal standard.

ranged from 88·5 to 188·7 ng/g and the B*a*P levels from 1·5 to 45·7 ng/g.

DISCUSSION

After some small modifications, the analytical procedure previously developed appeared to be sensitive and reliable as a routine method for the determination of B*a*P and other PAH in commercial suntan-oil samples of various types. This limited survey revealed the presence of low (ng/g) levels of carcinogenic, mutagenic, co-carcinogenic or non-carcinogenic PAH in the four products studied.

In particular, B*a*P was found in all the samples in a concentration range of 1·5–45·7 ng/g. Our previous study showed the presence of this carcinogenic PAH in purified liquid-paraffin samples, designated for medicinal and cosmetic use, at levels of 3·1–8·6 ng/g, and values reported in the literature for different vegetable oils have varied from 0 to 25 ng/g (Ciusa, D'Arrigo & Marchesini, 1970; Howard *et al.* 1966; Joe *et al.* 1979; Lintas *et al.* 1979; Monarca *et al.* 1981; Siegfried, 1975).

Anthanthrene, the other carcinogenic PAH identified (determined in only one sample, at the 1·2-ng/g level) was previously reported in vegetable oil in a 1·1-ng/g concentration (Joe *et al.* 1979).

Perylene, a non-carcinogenic PAH found at levels of 2·0–5·0 ng/g, was previously reported to be present in concentrations of 1·1 ng/g in a mineral oil (Monarca *et al.* 1981) and 12–60 ng/g in vegetable oils (Ciusa *et al.* 1970). Some authors have suggested that the potential of this compound as a carcinogen should be re-examined, since a recent study has shown that this PAH is a more potent mutagen than B*a*P (Penman, Kaden, Liber *et al.* 1980).

A relatively high concentration of the non-carcinogenic fluoranthene (35–151 ng/g, compared with 75·2–135·4 ng/g in our study) was also reported in vegetable oils by Ciusa *et al.* (1970). Fluoranthene showed co-carcinogenic activity when tested in combination with B*a*P using the molar ratio of the two compounds found in tobacco smoke (Hoffmann *et al.* 1978). Benzo[*k*]fluoranthene levels were close to those reported in the literature.

Other carcinogenic PAH, such as chrysene, benzo[*a*]anthracene, benzo[*e*]pyrene and benzo[*b*]fluoranthene, have previously been reported in mineral and vegetable oils, but were not found in the present study.

Our results suggest that users of suntan oils may be exposed to low levels of potentially hazardous PAH, but further studies of the PAH content of different suntan preparations will be necessary before conclusions can be drawn. Moreover, it is not known whether trace quantities of these compounds in suntan oils, combined with exposure to sunlight, could be a skin-cancer hazard, especially since these cosmetics are used only intermittently and for rather brief periods.

However, this preliminary study indicates the possibility of applying this analytical procedure to the determination of PAH levels in many other cosmetics, which are presumed to contain similar bases and are used more often and for longer periods.

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REPRODUCTION STUDY IN RATS OF GINSENG EXTRACT G115

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Abstract—The effect of ginseng extract G115 on reproductive performance was studied in two generations of Sprague–Dawley rats. Animals of both sexes were fed either control diet or diet supplemented with ginseng extract G115 at dose levels of 1·5, 5 or 15 mg/kg body weight/day. Parameters of reproduction and lactation in the treated groups were comparable to those of the controls for two generations of dams and pups. For F1 males and females, no treatment-related effects were seen in weekly body weights and food consumption, haematological and clinical chemical data, and ophthalmic, gross and histopathological examinations. The gross autopsies of F0 and F2 animals also revealed no significant treatment-related findings.

INTRODUCTION

In Chinese medicine, Oriental ginseng (*Panax ginseng*) has been used empirically as a tonic for protecting and preserving life. The perennial herb has been reported to have revitalization properties after a long illness (Leung, 1980), and has been claimed to be effective treatment for anaemia, rheumatism, sexual decline, constipation, diabetes, gastro-intestinal disorders, hypertension and stress (Chang, 1977). Many characteristics of its folklore capabilities have been supported by pharmacological studies in animals and humans.

Ginseng is used to maintain homeostasis under stressful situations (Farnsworth & Bederka, 1973). Its pharmacological activity apparently helps the body to adapt to various conditions of stress, by correcting adrenal and thyroid dysfunction (Siegel, 1979). Other properties of *P. ginseng* include a variety of general metabolic effects such as stimulation of liver gluconeogenesis, erythropoiesis, activity in the central nervous system, intestinal motility, muscular efficiency, and cell and tissue growth (Farnsworth & Bederka, 1973).

In the USA, ginseng is generally used in teas and in cosmetics, such as lotions, soaps and perfumes (Leung, 1980). The present investigation was carried out to evaluate the effects of feeding ginseng extract G115 at dose levels up to 15 mg/kg body weight/day on the reproductive performance of rats through two generations. [This dose level is greater than the recommended dose for man (Bittles, Fulder, Grant & Nicholls, 1979).]

EXPERIMENTAL

Animals and materials. Sprague–Dawley rats, weighing approximately 160–180 g at the beginning of the experiment, were obtained from Blue Spruce Farms, Altamont, NY. They were individually housed in an environment-controlled room maintained at 20–24°C and artificially illuminated for 12 hr/day. All animals were given Charles River RMH Formula 3200 diet (Agway, Waverly, NY) and tap water *ad lib.*

Ginseng extract G115, batch no. 950 126L, was supplied by GPL Ginseng Products Ltd, Lugano, Switzerland. G115 is the commercial designation of the standardized extract of *Panax ginseng* C. A. Meyer, produced by Pharmaton Ltd, Lugano-Bioggio, Switzerland.

Experimental design. The dose levels of ginseng extract G115 were 0, 1·5, 5 and 15 mg/kg body weight/day. The test diets were prepared by suspending ginseng extract G115 in corn oil and incorporating the mixture into rat feed using a Hobart blender. The amounts of ginseng extract G115 in the feed were adjusted weekly following determination of body weight and food consumption.

Each dose group consisted of 15 adult male and 15 adult female rats for the F0 generation. After 3 wk of feeding the respective diets, all of the rats in the F0 generation were paired, one male to one female within all of the dose groups. If insemination had not been confirmed by day 7, the female was paired with another male of the same group for an additional 6 days; if necessary, this procedure was repeated a third time. Females that did not become pregnant within this time were considered infertile.

Female rats of the F0 generation were fed test diets throughout mating, gestation and lactation. The F0 females were allowed to deliver normally to produce the F1 generation. The following parameters of reproductive performance were measured: number of pregnant females, number of pups born alive or dead, and survival of progeny.

Each litter (F1 generation) was randomly culled to eight pups (four males and four females for most litters) on day 4 *post partum*. The pups were weighed on the day of birth and on days 4 and 21 after birth. The litters were removed from the dams on day 21. Two

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males and two females, which were most representative of the mean body weight by sex within each litter, were selected from each litter (whenever possible) and used for a 13-wk subchronic feeding study and a further reproductive study. The remaining offspring and F0 parents were killed and gross autopsies were performed. The selected F1 animals were given the same levels of ginseng extract G115 as previously given to F0 animals. Ophthalmic examinations were conducted on each F1 animal at the initiation and termination of the subchronic study. After 6 and 13 wk of feeding, blood samples were collected from the periorbital plexus of five male and five female rats per group for determination of erythrocyte count, total and differential leucocyte counts, haematocrit, levels of haemoglobin, glucose, urea nitrogen, total protein, bilirubin, sodium, potassium, and chloride, and activities of serum glutamic–oxalacetic transaminase, serum glutamic–pyruvic transaminase and alkaline phosphatase.

After 13 wk of feeding, the F1 generation rats were paired, one male to one female within all of the groups. Female F1 rats were fed their respective test diet throughout mating, gestation and lactation. The F1-generation dams were allowed to deliver normally to produce the F2 generation. The parameters of reproductive performance measured were as previously described for the F0 generation. Each F2-generation litter was culled to eight pups (four males and four females for most litters) on day 4 *post partum*. When the F2 pups were 21 days old both they and the F1 animals were killed and autopsied.

Daily observations of physical appearance and behaviour, and weekly body weight and food consumption were recorded for the F0 and F1 generations. However, body weights and food consumption were not recorded for males and females during the mating period; they were recorded only for males during the weaning period.

Chloroform vapour was used to kill all of the animals. For F1-generation animals, selected organs (liver, spleen, thyroid gland, adrenal glands, heart and testes with epididymides or ovaries and uterus) were weighed. For the control and high-dose F1 animals, the organs or tissues that were taken for histopathological examination included the organs weighed as well as the following: brain, pituitary, spinal cord, eyes (with optic nerve), lungs, kidneys, stomach, pancreas, large and small intestine, urinary bladder, seminal vesicles, sciatic nerve/skeletal muscle, lymph nodes (mesenteric), skin (abdominal), sternum and bone marrow, grossly abnormal tissues. For the low- and middle-dose groups, only grossly abnormal tissues were examined.

Statistical methods. Analyses of body weight, food consumption, absolute and relative organ weights, and haematological and clinical chemical parameters were conducted using analysis of variance (Steel & Torrie, 1960). Differences between the test and control groups were determined using the least significant difference test. Discrete data were analysed using 2×2 contingency tables with Yates' correction for continuity (Bross, 1954).

RESULTS

For the F0 and F1 dams, neither the pregnancy rate (fertility index) nor the gestation, viability and lactation indices nor the pup mean body weights at days 0, 4 and 21 showed any significant trends associated with treatment (Table 1).

Gross autopsy examinations of the F0 animals revealed an abnormal change observed only in male test rats. Slightly swollen prostate glands were seen in several males of each treatment group, but the incidence was not dose-related. Therefore, no definite evidence of a morphological effect of the test material was observed.

The gross autopsies of F1 and F2 male and female rats showed no abnormal findings that were related to any treatment group. For F1 animals, no treatment-related effects were noted for absolute or relative organ weights. The microscopic examinations of tissues from F1 rats revealed no toxic or neoplastic morphological effects. Only one tumour was found; a mammary adenoma was observed in a female in the high-dose group.

For the 13-wk feeding study in F1 rats, analysis of weekly body weights showed no significant differences between treatment groups and the control group. For F1 male rats, mean initial body weights ($\pm$SEM) were 101 ± 2, 100 ± 2, 101 ± 2 and 101 ± 2 g for groups given dietary levels 0, 1·5, 5 and 15 mg/kg body weight/day, respectively. Mean final body weights were 397 ± 7, 398 ± 4, 412 ± 6 and 406 ± 7 g, respectively. For F1 female animals, initial mean body weights ($\pm$SEM) were 90 ± 2, 87 ± 1, 90 ± 2 and 89 ± 2 g and final body weights were 234 ± 3, 236 ± 3, 233 ± 4 and 234 ± 3 g for groups given dietary levels 0, 1·5, 5 and 15 mg/kg body weight/day, respectively.

Weekly food consumption data demonstrated no compound-related trends. The consumption of ginseng extract G115 had no effect on the haematological and biochemical parameters studied. Ophthalmic examinations of the F1 generation revealed no toxic effects of the test compound. No treatment-related effects on animal behaviour and physical appearance were noted for the F0 and F1 generations.

DISCUSSION

No significant evidence of toxicity or pathological effects was observed in the reproductive performance of two generations of male and female rats fed ginseng extract G115 at levels of up to 15 mg/kg body weight/day. The prostatic enlargement, observed grossly in several treated males of the F0 generation, was probably a random distribution of a slight variation in normal size. A treatment-associated trend was not noted.

The incidence of a single benign mammary gland tumour, which was seen in one female in the high-dose group, was not statistically significant. This type of neoplasm is a common spontaneous tumour in Sprague–Dawley female rats with a reported incidence of 23% (Benirschke, Garner & Jones, 1978).

In a review of previous animal studies (Popov & Goldwag, 1973), ginseng was reported to have very low acute and chronic toxicity. No toxic effects were noted in rats following ingestion of ginseng extract for 25 wk at daily dose levels of 105–210 mg/kg in the diet. In a subchronic feeding study of ginseng extract

Table 1. *Reproductive performance of two generations of female rats fed diets containing ginseng extract G115*

Dietary level (mg/kg body weight/day)	No. of mated females (no. pregnant)	No. of pups per litter*		Indices†				Body weights of pups aged*			
										21 days	
		Born alive	Born dead	Fertility	Gestation	Viability	Lactation	0 days	4 days	Male	Female
F0 generation											
0	15 (15)	11·9 ± 0·6	0·2 ± 0·1	100	100	98	98	6·3 ± 0·1	10·8 ± 0·3	50·5 ± 1·2	46·9 ± 1·3
1·5	15 (13)	13·2 ± 0·6	0·1 ± 0·1	87	100	99	100	6·3 ± 0·1	9·9 ± 0·3	50·0 ± 0·9	46·9 ± 0·9
5	15 (11)	12·0 ± 0·6	0·2 ± 0·2	73	100	100	99	6·2 ± 0·1	10·5 ± 0·3	49·3 ± 1·2	47·0 ± 1·2
15	15 (13)	12·4 ± 0·4	0·2 ± 0·2	87	100	99	100	6·2 ± 0·2	10·2 ± 0·3	50·7 ± 1·4	47·0 ± 1·2
F1 generation											
0	30 (29)	11·3 ± 0·5	0·2 ± 0·1	97	100	98	94	6·3 ± 0·1	10·3 ± 0·2	48·5 ± 0·9	45·9 ± 0·8
1·5	30 (30)	11·7 ± 0·4	0·4 ± 0·2	100	100	97	96	6·1 ± 0·1	10·2 ± 0·3	50·2 ± 1·0	47·1 ± 0·8
5	30 (28)	12·5 ± 0·3	0·2 ± 0·1	93	100	97	95	6·1 ± 0·1	9·8 ± 0·2	49·3 ± 1·0	47·0 ± 0·9
15	30 (29)	11·9 ± 0·4	0·1 ± 0·1	97	100	98	93	6·2 ± 0·1	9·7 ± 0·3	50·8 ± 0·9	47·4 ± 1·0

*Values are means ± SEM for the number of litters or pups indicated.

†The indices were calculated as follows: fertility index—percentage of mated females that became pregnant; gestation index—percentage of pregnancies resulting in litters born alive; viability index—percentage of pups born alive that survived to 4 days of age; lactation index—percentage of pups alive at 4 days that survived to 21 days of age [each litter was culled to eight pups (four males and four females for most litters) on day 4 *post partum*].

G115 in beagle dogs, no significant evidence of toxicity or pathological effects was noted (F. G. Hess, P. J. Becci, K. R. Stevens, G. E. Cox & R. A. Parent, unpublished data, 1981). In a previous experiment involving stress responses in mice, stimulation of behavioural reactions to mild stress was caused by ginseng extract dissolved in drinking-water at a dose level of 8 mg/kg body weight/day (Bittles *et al.* 1979).

In the present study, the high dose level of 15 mg extract/kg body weight/day produced no pathological or toxic effects. Further animal studies and clinical trials may clarify the known properties of ginseng and provide for new applications.

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pH AND THE POTENTIAL IRRITANCY OF DOUCHE FORMULATIONS TO THE VAGINAL MUCOSA OF THE ALBINO RABBIT AND RAT*

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Abstract—Studies were conducted to determine the effect of the pH of a vaginal douche on its irritation potential for the vaginal mucosa of the albino rabbit and albino rat. The pH of a liquid douche formulation was varied by modification of the lactic acid/sodium bicarbonate ratio and the resulting preparations were administered intravaginally by lavage once a day for four consecutive days. On day 5, the animals were killed and the vaginal tissues were processed and examined histopathologically. Using a predetermined semiquantitative scoring system, it was found that the irritation potential of the formulation under study was pH dependent in the rabbit but not in the rat. As the pH was decreased, the degree of irritation became more severe in the rabbit; at pH 3·0 and below, irritation was sufficiently severe for the formulation to be considered unacceptable for human use. Only minimal irritation was seen in the rat at any of the pH levels tested. The sensitivity of the rabbit vaginal mucosa is greater than that of the rat and the response is pH dependent below pH 3·0. This exaggerated response must be taken into consideration when the rabbit is used for assessing the vaginal-irritation potential of douches. The rabbit, having a vaginal mucosa more sensitive than that of the rat, remains an appropriate model for measuring the potential irritancy of new douche products, because comparisons can be made with products of known irritancy.

INTRODUCTION

Cosmetic douches continue to be popular, and are used by a considerable portion of the female population. It is essential, therefore, that safety testing be undertaken prior to marketing and that the potential for local irritation of the vaginal mucous membranes be acceptable. Screening these products in animals is an appropriate method for evaluating vaginal irritation potential, and the rabbit vagina is routinely used (Eckstein, Jackson, Millman & Sobrero, 1969). Comparison of the findings in vaginal studies in the rabbit with those in clinical studies of the human vagina has demonstrated good agreement between the rabbit and human with regard to the vaginal irritation potential of spermicidal preparations (Eckstein *et al.* 1969).

The mucous membrane of the rabbit vagina differs from that of the human vagina in that it does not have an outer cornified epithelial layer. In contrast, the rat vaginal mucous membrane, like that of the human, has a cornified epithelial layer and therefore would be expected to provide a response more representative of the human tissue. The normal pH of the human vagina has been reported to be approximately 4·0 (FDA Advisory Review Panel, 1978). In this laboratory the vagina of the New Zealand albino rabbit was found to have an average pH of 7·5 and the vagina of the Sprague–Dawley rat an average of 7·0. These differences in pH between the rabbit, rat and human vagina prompted the studies reported here.

EXPERIMENTAL

Test materials. A liquid douche formulation containing lactic acid and sodium bicarbonate in ratios adjusted to yield various pH values was used for all the tests.

Animals. Virgin female New Zealand albino rabbits, weighing between 2·3 and 4·0 kg, and virgin female Sprague–Dawley caesarean-derived albino rats weighing 200–300 g were acclimatized to laboratory conditions for at least 1 wk before use. Both species were housed individually in wire-mesh cages in rooms controlled for temperature on a 12-hr light/dark cycle. The animals, separated by species, were provided with a standard laboratory diet (Purina Rabbit or Rodent Chow, from Ralston Purina Co. Inc., St. Louis, MO) and water *ad lib.*

Experimental procedures

Prior to the start of the study the general physical appearance of each animal was determined and the vaginal mucosa of each animal was examined for gross abnormalities. Any animal exhibiting abnormalities in physical appearance or in the vaginal mucosa was replaced. Three separate experiments were conducted.

* A preliminary report of these investigations was presented on 4 March 1981 at the 20th Annual Meeting of the Society of Toxicology, held in San Diego, CA.

Experiment I. Four groups, each of six rabbits, were dosed with the douche formulation at pH 7·5, 6·0, 4·0 or 3·3. A fifth group served as a control and received only distilled water. By means of a syringe and rubber catheter 20 ml of test material was introduced by lavage into the vaginal vault. The rubber catheter, lubricated with K-Y jelly (Johnson & Johnson, New Brunswick, NJ) was inserted into the vaginal vault to a depth of 90–120 mm. The test material was released over 40–50 mm while the catheter was slowly withdrawn. This treatment was repeated once daily for four consecutive days. The test formulation was at the normal in-use concentration and the volume was sufficient to lavage the vaginal vault completely, exposing the entire vaginal mucosal surface.

Experiment II. Six groups each of six rabbits were dosed with the douche formulation at pH 4·0, 3·5, 3·0, 2·5 or 2·0 or with distilled water (control group). The test material was administered in the same manner as in Experiment I.

Experiment III. Six groups each of eight rats were dosed with the douche formulation at pH 4·0, 3·6, 3·2, 2·8 or 2·3 or with distilled water (control group). A volume of 0·5 ml was introduced in the same manner as in the rabbit, except that the catheter was inserted into the vaginal vault to a depth of 20 mm. The catheter was withdrawn slightly while the barrel of the syringe was pressed to release the contents, lavaging and exposing the entire surface of the vaginal mucosa. The treatment schedule was the same as that used in the two rabbit experiments.

Observations. On the day following the last treatment, the animals from each experiment were killed by carbon dioxide suffocation. The vagina of each animal was removed, slit open longitudinally, spread out and, with the mucosa facing upward, stapled to an index card. Following fixation for 1 wk in 10% formalin, three sections of each vagina (cervical end, middle and vulvar end) were embedded in paraffin, sectioned and stained with standard haematoxylin and eosin for histopathological examination. The degree of irritation was determined according to the method described by Eckstein *et al.* (1969). The parameters examined and scored were epithelial exfoliation, haemorrhage, oedema, mucosal necrosis and inflammatory-cell infiltrate. The scoring system used for the degree of response was as follows: 1 = minimal, 2 = slight, 3 = moderate and 4 = marked. The scores for the animals in each group were totalled and averaged.

RESULTS

Experiment I

At all four pH values the test douche was more irritating than the distilled-water control to the vaginal mucosa of the albino rabbit (Table 1). The average vaginal-irritation scores were comparable for the three higher pH values tested and the score produced by the test douche at pH 3·3 was approximately twice as great as the scores for the douche at pH 4·0, 6·0 or 7·5 (Table 1). Marked epithelial exfoliation was seen in each of the test groups, but was not seen in the control group (Table 2). Submucosal haemorrhage was observed consistently only in the pH 3·3 group, while submucosal oedema was observed in several animals in each test group but was not observed in the control animals (Table 2). Mucosal necrosis and acute inflammatory cell infiltrate were not observed in either test or control groups.

Experiment II

The douche formulations at pH 4·0, 3·5 and 3·0 produced comparable average vaginal irritation scores, which were greater than that produced by distilled water (Table 1). The scores produced by the douche at pH 2·5 and 2·0 were, respectively, about two and three times greater than the scores at pH 4·0, 3·5 and 3·0 (Table 1). The histological findings are summarized in Table 3. Mucosal necrosis was persistent and intense in the pH 2·0 and 2·5 test groups, showed isolated occurrence in the pH 3·0 group and was not seen in the pH 3·5 and pH 4·0 groups or in the control animals. Submucosal haemorrhage was observed in the pH 2·0, 2·5 and 3·0 groups, but not in the pH 3·5, 4·0 and control groups. Inflammatory cell infiltrate was observed in each of the test groups except the pH 3·0 group, and epithelial exfoliation was persistent and intense in each of the test groups, but neither effect was seen in the control group.

Experiment III

The average vaginal irritation scores produced by the douche at each of the pH values tested in the albino rat were minimal and similar to that of the control group (Table 1). Inflammatory-cell infiltrate was observed in both the control and test groups, except for the pH 4·0 group, but was only minimal. Epithelial exfoliation, haemorrhage, submucosal oedema and mucosal necrosis were not seen in any of the test groups or in the control group.

Table 1. *Mean vaginal-irritation scores in rabbits and rats following administration of vaginal douches varying in pH*

Experiment	pH of formulation	Mean score*
	Rabbit studies	
I	3·3	8·5
	4·0	4·3
	6·0	3·9
	7·5	4·5
	(Control)	0·0
II	2·0	20·7
	2·5	14·7
	3·0	6·5
	3·5	7·7
	4·0	7·8
	(Control)	1·7
	Rat study	
III	2·3	0·1
	2·8	0·3
	3·2	0·4
	3·6	0·4
	4·0	0·0
	(Control)	0·3

*Values are means for groups of six rabbits or eight rats, the degree of irritation being determined for individual animals by the method of Eckstein *et al.* (1969).

Table 2. *Microscopic observations within the vagina of rabbits exposed intravaginally to douche formulations of pH 3·3–7·5 (experiment I)*

Exposure	Animal no.	Histopathological response*		
		Epithelial exfoliation (mucosa)	Haemorrhage (submucosa)	Oedema (submucosa)
Control	1	—	—	—
	2	—	—	—
	3	—	—	—
	4	—	—	—
	5	—	—	—
	6	—	—	—
pH 3·3	7	4	2	3
	8	4	1	—
	9	4	2	—
	10	4	3	4
	11	4	3	4
	12	4	2	3
pH 4·0	13	4	—	2
	14	4	—	3
	15	4	—	1
	16	4	—	—
	17	(4)	—	—
	18	—	—	—
pH 6·0	19	4	—	—
	20	4	—	—
	21	(4)	—	—
	22	—	—	—
	23	(4)	—	—
	24	4	—	3
pH 7·5	25	—	—	—
	26	4	—	—
	27	4	—	3
	28	4	—	—
	29	4	1	3
	30	4	—	—

*Numerical grading of response: 1 = minimal; 2 = slight; 3 = moderate; 4 = marked; – = no changes apparent (i.e. tissues within normal histological limits); ( ) = isolated or occasional change.

DISCUSSION

These studies evaluated the effect of the pH of a vaginal douche on its irritation potential for the vaginal mucosa in the albino rabbit and the albino rat. The pH of the douche influenced its irritation potential in the rabbit but not in the rat. In the first experiment, the average vaginal-irritation score for the douche tested down to pH 4·0 was in the acceptable range (0–8) according to the criteria used by Eckstein *et al.* (1969). This irritation showed a significant increase at pH 3·3, a difference due primarily to the occurrence of submucosal haemorrhage. In the second experiment, which was conducted for the purpose of examining the effects of pH values lower than 4·0, where irritation was expected to be most severe, average vaginal irritation scores were in the acceptable range down to a pH of 3·0. The scores produced by the douche below this pH were in the range regarded as unacceptable for human use (above 10). This increase in irritation was due primarily to the occurrence of submucosal haemorrhage and mucosal necrosis, which were seen at pH 3·0 and increased as the pH decreased.

The third experiment was conducted to examine the effect of the pH of the douche on the vaginal mucosa of the albino rat. The irritation produced by the douche formulation was minimal and was comparable for all the pH values tested from 4·0 to 2·3 and for distilled water. Application of the criteria established by Eckstein *et al.* (1969) for rabbits placed the average vaginal irritation scores in the acceptable range (0–8).

These findings show that the vaginal mucosa of the rabbit is more sensitive to irritation than is that of the rat. The sensitivity in the rabbit is pH dependent, increasing at pH values below 3·0. The use of the rabbit may give an exaggerated representation of the irritation potential to the human vagina of formulations with a pH below 3·0. The human vagina normally has a lower pH than does the rabbit vagina and it has a cornfield epithelial mucosal lining, which is found also in the rat but not in the rabbit. The human vaginal mucosa, therefore, may be less sensitive to

Table 3. *Microscopic observations within the vagina of rabbits exposed intravaginally to douche formulations of pH 2·0–4·0 (experiment II)*

Exposure	Animal no.	Histopathological response*				
		Epithelial exfoliation (mucosa)	Haemorrhage (submucosa)	Oedema (submucosa)	Mucosal necrosis	Acute neutrophil infiltrate
Control	1	—	—	2	—	—
	2	—	—	—	—	—
	3	—	—	3	—	—
	4	—	—	1	—	—
	5	—	—	2	—	—
	6	—	—	2	—	—
pH 2·0	7	4	3	4	4	3
	8	4	4	4	4, 4†	4
	9	4	4	4	4, 4†	3
	10	4	4	4	4	2
	11	4	4	4	4, 3†	3
	12	4	4	4	4	2
pH 2·5	13	4	3	2	4	2
	14	4	3	3	4	2
	15	4	2	2	4	1
	16	4	2	2	4	2
	17	4	2	3	4	1
	18	4	1	3	4	1
pH 3·0	19	4	—	3	—	—
	20	4	2	3	(2)	—
	21	—	—	2	—	—
	22	4	1	1	—	—
	23	4	2	—	—	—
	24	4	—	3	—	—
pH 3·5	25	4	—	2	—	—
	26	4	—	3	—	—
	27	4	—	3	—	3
	28	4	—	3	—	1
	29	4	—	3	—	—
	30	4	—	3	—	1
pH 4·0	31	4	—	2	—	2
	32	4	—	3	—	1
	33	4	—	3	—	2
	34	4	—	3	—	1
	35	4	—	3	—	—
	36	4	—	3	—	—

*Numerical grading of response: 1 = minimal; 2 = slight; 3 = moderate; 4 = marked; — = no changes apparent (i.e. tissues within normal histological limits); ( ) = isolated or occasional change.
†Vulva response.

acidic pH and, therefore, better able to tolerate these formulations. Its response may resemble more closely that of the rat than that of the rabbit.

The irritation of the vaginal mucosa of the albino rabbit and the albino rat by douches of low pH still remains to be correlated with the irritation of human vaginal tissue before definite conclusions can be drawn.

The rabbit, having a vaginal mucosa that is more sensitive than that of the rat, remains an appropriate animal model for measuring the potential irritancy of new douche products, because comparisons can be made with products of known irritancy.

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EFFECTS OF LACTOBACILLUS, ANTACIDS AND ANTIBIOTICS ON THE LEVELS OF NITRITE IN THE GASTRO-INTESTINAL TRACTS OF RATS FED SODIUM NITRATE

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Abstract—No nitrite was detected in the tissues or contents of the gastro-intestinal tracts of normal rats but after 2 wk on a diet containing 0·5% sodium nitrate the levels of nitrite in the stomach, small intestine and large intestine contents were 0·83, 1·64–2·07 and 0·53–0·83 μg/g of contents respectively. Concurrent administration of 2% Lactobacillus preparation and 0·5% sodium nitrate in the diet for 2 wk further increased the nitrite levels in the intestines and slightly increased the level in the stomach, The elevation of nitrite levels induced by sodium nitrate administration was potentiated considerably by combined treatment with sodium bicarbonate and hetacillin producing nitrite levels of 3·16, 2·93–5·18 and 1·96–2·34 μg/g of the contents of the stomach, small intestine and large intestine respectively. Like hetacillin, minomycin and thiamphenicol also potentiated the nitrite production whereas amikacin (another antibiotic) strongly inhibited the formation of nitrite in the stomach. The different effects of the antibiotics may be due to their selective activities on the various microbes. The results indicate that the levels of nitrite in the gastro-intestinal tract are regulated by the level of nitrate intake, the population of microflora and the gastric pH. The safety of combined medication with antacids, antibiotics and Lactobacillus preparations in man deserves further investigation.

INTRODUCTION

In recent years, a great number of *N*-nitroso compounds have been shown to be carcinogenic to various experimental animals (Magee & Barnes, 1967), and most organs are sensitive to the carcinogenic action of these compounds (Druckrey, Preussman & Ivankovic, 1969). The presence of nitrate, nitrite and *N*-nitroso compounds in food has become a matter of great concern because of their potential and actual toxic and carcinogenic characteristics (Lijinsky & Epstein, 1970). Nitrite is known to react with secondary or tertiary amines to form carcinogenic *N*-nitroso compounds and a number of investigators have attempted to assess the possible role of nitrate and nitrite in the aetiology of human cancer (Swan, 1975; Walters, Carr, Dyke *et al.* 1979).

Several vegetables such as spinach (Aworh, Brecht & Minotti, 1978), beets (Heisler, Siciliano, Krulick *et al.* 1974), cabbages and mustards (Lin & Lue, 1979) can accumulate high levels of nitrate, which may be reduced to nitrite during storage. The effect of nitrate intake on the formation of salivary nitrite in man has been studied and the extent of nitrite formation is related to the level of nitrate intake and to the oral microflora population (Tannenbaum, Sinskey, Weisman & Bishop, 1974; Tannenbaum, Weisman & Fett, 1976). Several studies have demonstrated that parotid duct saliva is free of nitrite, and that a major route of nitrite formation is the microbial reduction of the nitrate naturally present in saliva. Furthermore, nitrite and nitrate are synthesized in the human intestine (Tannenbaum, Fett, Young *et al.* 1978). These findings suggest an even wider role for nitrite in the aetiology of human cancer (Tannenbaum, Moran, Rand *et al.* 1979).

Patients with achlorhydria have an increased susceptibility to gastric cancer (Ruddell, Bone, Hill *et al.* 1976; Segal & Samloff, 1973). The stomach is sterile in most fasting normal people; only in patients with gastric achlorhydria is bacterial colonization of the stomach seen. Gastric acidity apparently influences the passage of bacteria from the mouth to the intestine; in a group of people with normal levels of gastric acid, the upper small intestine was virtually free from bacteria except after meals (Drasar, Shiver & McLeod, 1969). From these observations, it seems that there are several factors, including the intake of nitrate, the population of microbes and the pH of the gastric contents, that determine the concentration of nitrite in the stomach and intestines. This study was designed to elucidate the possible mechanism of nitrite accumulation in the gastro-intestinal tract of rats.

EXPERIMENTAL

Lactobacillus preparation. The pharmaceutical preparation, Noramine, was purchased from the local drug store here in Taipei. This kind of Lactobacillus preparation is sold under several trade names, among which Noramine is the most popular. It contained viable *Lactobacillus acidophilus*, *L. bulgaricus*, *Streptococcus faecalis* and *Bacillus natto* as its active principles. Noramine is claimed by its manufacturer to be effective in the treatment and prevention of various

Abbreviations: GIT = Gastro-intestinal tract.

digestive disorders, particularly enteritis, diarrhoea and dyspepsia. This preparation has been shown to possess nitrate reductase activity (Lin & Lai, 1979).

Antibiotics. The following antibiotics were used: Hetacillin potassium (Bristol, Syracuse, NY, USA), penicillin G potassium (Yong-Fong, Taipei), cephalexin monohydrate (Lilly, Indianapolis, IN, USA), oxytetracycline (Pfizer, New York, USA), minomycin (Lederle, Wayne, NJ, USA), tetracycline (Hoechst, Frankfurt, West Germany), kanamycin sulphate (Tsing-Tung, Tao-Yen), streptomycin sulphate (Yong-Fong), chloramphenicol (Chung-Kuo, Su-lin), thiamphenicol (Zambon, Minolta, Italy), erythromycin estolate (Lilly), colistin sulphate (Warner-Chilcott, Morris Plains, NJ, USA), clindamycin (Upjohn, Kalamazoo, MI, USA) and amikacin (Bristol).

Chemicals. Sodium dithionite, sodium nitrate, sodium nitrite, sodium bicarbonate and α-naphthylamine were purchased from E. Merck Co., Darmstadt, Germany. Calcium carbonate and sulphanilic acid were purchased from Wako Chemical Co., Tokyo. Methyl viologen was purchased from Sigma Chemical Co., St. Louis, MO, USA.

Animal experiments. Male albino Sprague–Dawley rats weighing 250–300 g were used. The experimental animals were given nitrate in the diet; the microbe populations in these animals were manipulated by administration of Lactobacillus or antibiotics; meanwhile, the pHs of their stomachs were altered by giving antacids such as $NaHCO_3$ or $CaCO_3$.

The basal diet comprised wheat flour (2150 g), rice bran skin (370 g), casein (150 g), salt mixture (45 g), fish liver oil (30 ml) and peanut oil (270 ml). The salt mixture was prepared by mixing $CaCO_3$ (16 g), K_2HPO_4 (44 g) and NaCl (40 g). The test diets were prepared by mixing basal diet with 2% Lactobacillus preparation, 0·5% sodium nitrate, 0·5–2·4% sodium bicarbonate and/or 0·05% hetacillin (final concentrations in the diet).

The animals were divided into groups of three to five rats. The control group was given the basal diet while the test groups were given various treatments as indicated in the tables. Each group of animals was housed in a stainless steel wire cage and food and water were provided *ad lib.* Most experiments were ended after 2 wk although some lasted 10 wk; the animals were killed and the GITs were removed immediately for nitrite estimation and nitrate-reductase assay.

The GITs of rats were divided into stomach, upper small intestine, lower small intestine, upper large intestine and lower large intestine. Both intestines were divided equally into upper and lower portions. Each segment was weighed rapidly and washed with 10 ml of cold distilled water. The washing containing GIT contents including ingested foods, microbes, enzymes, mucins, electrolytes, water, etc. were used directly for the estimation of nitrite and nitrate reductase. The washed GIT tissues were pressed gently with Watman No. 1 filter paper to remove the excess water and were weighed and homogenized with 0·9% sodium chloride to give a 10% homogenate in which the concentration of nitrite and the activity of nitrate reductase were estimated as described below. In some experiments the intact GIT segments including tissues and contents were cut into pieces and homogenized with deionized water to give a 10% homogenate.

Estimation of nitrite. The homogenate from either intact GIT or GIT tissue was centrifuged at 2000 g for 15 min and the supernatant (1 ml) was then mixed with 1 ml acetone. The mixture was again centrifuged and 1 ml of the resulting clear supernatant was mixed with 0·5 ml of 2% sulphanilic acid in 0·2 M–acetic acid and 0·5 ml of 0·8% α-naphthylamine in 0·2 M–acetic acid. The reaction mixture was allowed to stand at room temperature (25–27°C) for 30 min. The pink substance thus formed was extracted with 5 ml of *n*-butanol and estimated spectrophotometrically at 520 nm in a Shimadzu UV-200S double beam spectrophotometer (AOAC, 1975). Under these experimental conditions, the recoveries of added nitrite (1 μg/g) from the stomach, small intestine and large intestine were found to be 90–96%, 82–86% and 75–80%, respectively. The standard deviations of the recovery experiments were within 4% of the mean and were less than the variations in the different GIT samples. The detection limit was found to be around 0·02 μg/g of sample.

Assay for nitrite reductase. The activity of nitrate reductase was estimated by the method of Lowe & Evans (1964) with some modifications. The bacterial population was estimated spectrophotometrically at 575 nm against distilled water. The assay mixture contained in a final volume of 2 ml, potassium dihydrogen phosphate (0·03 M, pH 7·0), methyl viologen (0·004%), sodium dithionite (4·6 mM), sodium bicarbonate (9·6 mM), sodium nitrate (0·02 M) and a specified amount of the tissue homogenate or bacterial suspension (1 ml). A sample containing the same amount of tissue was heated for 2 min in a boiling water bath before the addition of substrate and served as the reaction blank. The reaction mixture was incubated at 37°C for 20 min, and the reaction was linear with respect to time and enzyme concentration during this incubation period. The reactions were stopped by vigorous aeration until the blue colour had com-

Table 1. *Nitrate reductase activity in the rat gastro-intestinal tract*

	Nitrate reductase (μg nitrite/g of GIT tissue or content/min at 37°C)				
		Small intestine		Large intestine	
Source	Stomach	Upper	Lower	Upper	Lower
GIT tissue	0·25 ± 0·007	0·32 ± 0·02	0·44 ± 0·02	0·33 ± 0·03	0·30 ± 0·02
GIT contents	0·07 ± 0·005	0·29 ± 0·02	0·15 ± 0·008	0·09 ± 0·004	0·04 ± 0·004

GIT = Gastro-intestinal tract

Values are means ± SEM for five determinations.

pletely disappeared and then 2 ml of 2% acetic acid in acetone was added to precipitate the protein. The mixture was centrifuged at 2000 **g** for 15 min and 1 ml of the supernatant was removed for nitrite determination as described above. The activity of nitrate reductase was calculated and expressed as μg nitrite formed/g of tissue or content or/unit absorbance at 575 nm (for microbes)/min.

Induction of nitrate reductase. The induction of nitrate reductase in the presence of sodium nitrate in *E. coli* or isolated gastric microflora and the effects of 14 kinds of antibiotics on this induction were studied both in synthetic medium and 0·85% NaCl. The synthetic medium consisted of 0·4% NH_4Cl, 0·01% $MgSO_4$, 0·3% KH_2PO_4, 0·6% Na_2HPO_4, 0·4% glucose, 0·05% $NaNO_3$ and the various concentrations of antibiotics indicated in the table. The concentrations of the antibiotics were derived from their effective therapeutic dosages as cited in the United States Pharmacopeia (1975) and related literature.

E. coli B from stock culture or microbes freshly isolated from rat stomachs or intestinal tracts were transferred aseptically to the synthetic medium or to 0·85% NaCl containing 0·05% $NaNO_3$. The cultures were incubated at 37°C for 2 days. The growth of the microbes was quantitated by cell-density–absorbance relationship at 575 nm against distilled water. The microbial cells were harvested by centrifugation at 2000 **g** for 15 min, washed with deionized water twice, and finally used for nitrate reductase assay.

RESULTS

Nitrate reductase activity in normal rat GIT

Nitrate reductase activity was detected in all segments of the rat GIT in both tissues and contents (Table 1). Of the tissues the lower segment of small intestine showed the highest enzyme activity while the stomach showed the lowest enzyme activity. In general the specific activity of the enzyme was higher in GIT tissues than in GIT contents. Whether the enzyme activity detected in the GIT tissues originated from the mammalian cells themselves or from the inhabiting microbes is unknown at the present.

Effects of Lactobacillus preparation and sodium nitrate on nitrite formation

No nitrite was detected in the GIT contents or tissues of control animals (Table 2). Administration of $NaNO_3$ increased the nitrite level in the GIT contents particularly in the upper segment of small intestine (Table 2). Concurrent administration of Lactobacillus preparation and $NaNO_3$ significantly increased the nitrite concentration in the contents of the large intestine and upper small intestine and only increased slightly the concentration in the stomach (Table 2). Under the treatment of Lactobacillus, the elevation of gastric and intestinal nitrite was dependent on the concentration of sodium nitrate (J.-K. Lin and C.-C. Lai, unpublished data, 1981). Administration of sodium nitrate enhanced the level of nitrite in the tissues of the stomach and large intestine, whereas no appreciable effect was detected in the small intestine (Table 2). The levels of nitrite in GIT tissues were fairly stable and were not significantly altered by the Lactobacillus preparation (Table 2).

In 10-wk experiments, similar results were obtained, but the extent of elevation in the concentration of nitrite was somewhat less than that in the 2-wk experiments (data not shown).

Effects of antacids and antibiotics on the level of nitrite

The effects of sodium bicarbonate, calcium carbonate and hetacillin on the level of nitrite in the GIT contents of rats fed sodium nitrate are summarized in Table 2. Sodium bicarbonate alone had no effect on gastro-intestinal nitrite (data not shown). In combination with sodium nitrate, sodium bicarbonate slightly increased the nitrite level in the gastric contents and significantly increased the nitrite level in the contents of both small and large intestines; the Lactobacillus preparation potentiated these effects (Table 2). Another antacid, calcium carbonate, produced similar but less significant increases in nitrite levels, probably because of its poor solubility (Table 2).

Hetacillin alone had no effect on the concentration of nitrite in the GIT contents. In combination with sodium nitrate, the antibiotic increased the level of nitrite in the contents of the stomach and large intestine and to a lesser extent in the contents of the upper small intestine; when the treatment also included sodium bicarbonate the antibiotic potentiated the elevation of the nitrite levels in the contents of all GIT segments (Table 2).

Another series of experiments was carried out to estimate the total amount of nitrite in both the tissues and contents of the GIT of rats fed 0·5% sodium nitrate for 2 wk (Table 3). Combined treatment with hetacillin and sodium bicarbonate significantly enhanced the level of nitrite in each of the gut segments.

It became clear from our experiments on the effects of Lactobacillus, antibiotics and antacids on the formation of nitrite in the GIT, that the stomach and the upper small intestine were the major sites of nitrite enhancement. Additional experiments were designed and performed to investigate further these effects. The effects of the antibiotics, hetacillin, oxytetracycline, minomycin, streptomycin, chloramphenicol, amikacin and thiamphenicol in combination with sodium bicarbonate on the nitrite formation in the stomachs of rats fed $NaNO_3$ are summarized in Table 4. Hetacillin, minomycin and thiamphenicol increased the level of stomach nitrite to a highly significant extent, whereas oxytetracycline, streptomycin and chloramphenicol failed to do so. Amikacin produced a highly significant decrease in the level of gastric nitrite.

Effects of various treatments on nitrate reductase activity in rat GIT contents in vivo

In these experiments the GIT contents of treated rats were isolated aseptically and transferred to the synthetic medium containing 0·05% sodium nitrate and incubated at 37°C for 2 days. The preliminary studies indicated that pre-incubation of the GIT contents with nitrate increased the activity of nitrate reductase (cf. Tables 1 & 5). Although the enzyme activity thus obtained does not represent the situation in the intact intestine it should indicate the potential enzyme activity of each GIT segment if nitrate were present in the diet.

The activities of nitrate reductase in the GIT con-

Table 2. *Effects of sodium bicarbonate, calcium carbonate, Lactobacillus preparation and hetacillin on the level of nitrite in the GIT contents or tissues of rats fed sodium nitrate*

		Formation of nitrite (μg/g of GIT content or tissue)			
		Small intestine		Large intestine	
Treatment†	Stomach	Upper	Lower	Upper	Lower
		GIT contents			
Control, no treatment	ND	ND	ND	ND	ND
$NaNO_3$	0·83 ± 0·11	2·07 ± 0·32	1·64 ± 0·17	0·83 ± 0·11	0·53 ± 0·14
$NaNO_3$, $NaHCO_3$	0·92 ± 0·04	5·09 ± 0·88**	1·84 ± 0·36	1·39 ± 0·25*	0·61 ± 0·12
$NaNO_3$, Lactobacillus	1·09 ± 0·13	3·58 ± 0·70*	1·71 ± 0·40	1·28 ± 0·21*	0·91 ± 0·12*
$NaNO_3$, Lactobacillus, $CaCO_3$	1·22 ± 0·07**	4·25 ± 0·38***	2·57 ± 0·40*	1·62 ± 0·25*	0·84 ± 0·12
$NaNO_3$, Lactobacillus, $NaHCO_3$	1·71 ± 0·16***	5·28 ± 0·51***	2·05 ± 0·35	1·78 ± 0·30**	1·61 ± 0·21***
$NaNO_3$, Hetacillin	1·61 ± 0·18***	4·18 ± 0·70*	2·12 ± 0·23	2·13 ± 0·08***	1·89 ± 0·37***
$NaNO_3$, Hetacillin, $NaHCO_3$	3·16 ± 0·18***	5·18 ± 0·54***	2·93 ± 0·12***	1·96 ± 0·25***	2·34 ± 0·18***
		GIT tissues			
Control, no treatment	ND	ND	ND	ND	ND
$NaNO_3$	0·57 ± 0·05	ND	ND	0·31 ± 0·04	0·25 ± 0·03
$NaNO_3$, $NaHCO_3$	0·62 ± 0·06	ND	ND	0·32 ± 0·04	0·23 ± 0·02
$NaNO_3$, Lactobacillus, $NaHCO_3$	0·70 ± 0·04*	ND	ND	0·38 ± 0·03	0·27 ± 0·03
$NaNO_3$, hetacillin, $NaHCO_3$	0·75 ± 0·09	ND	ND	0·40 ± 0·03	0·25 ± 0·03

GIT = Gastro-intestinal tract ND = Not detected

†The concentrations of the treatment chemicals in the diet were $NaNO_3$, 0·5%, $NaHCO_3$, 0·5%, $CaCO_3$, 0·5%, Lactobacillus preparation, 2% and hetacillin, 0·05% throughout the experiment.

Values are means ± SEM for five determinations; those that differ significantly (Student's *t* test) from the corresponding values for the nitrate only treatment are marked with asterisks (*$P < 0·05$; **$P < 0·01$; ***$P < 0·005$).

Table 3. *Effects of sodium bicarbonate, Lactobacillus preparation and hetacillin on the level of nitrite in the GIT (tissues and contents combined) of rats fed sodium nitrate*

Treatment†	Formation of nitrite (μg/g of GIT tissue and contents combined)					Total nitrite in GIT (μg)
	Stomach	Small intestine		Large intestine		
		Upper	Lower	Upper	Lower	
Control, no treatment	ND	ND	ND	ND	ND	ND
$NaNO_3$	0·45 ± 0·06	0·50 ± 0·07	0·34 ± 0·04	0·26 ± 0·09	0·15 ± 0·02	4·68 ± 0·97
$NaNO_3$, $NaHCO_3$	0·57 ± 0·04	0·93 ± 0·09***	0·50 ± 0·08	0·27 ± 0·04	0·15 ± 0·03	—
$NaNO_3$, Lactobacillus	0·58 ± 0·06	0·72 ± 0·09*	0·41 ± 0·09	0·25 ± 0·05	0·19 ± 0·03	5·93 ± 1·07
$NaNO_3$, Lactobacillus, $CaCO_3$	0·69 ± 0·04**	1·08 ± 0·11***	0·53 ± 0·05**	0·27 ± 0·04	0·23 ± 0·03*	—
$NaNO_3$, Lactobacillus, $NaHCO_3$	0·84 ± 0·07***	1·18 ± 0·11***	0·64 ± 0·22	0·49 ± 0·05*	0·36 ± 0·06***	7·50 ± 1·42
$NaNO_3$, hetacillin	0·78 ± 0·07***	1·10 ± 0·11***	0·50 ± 0·04**	0·46 ± 0·04**	0·37 ± 0·07**	8·45 ± 1·25*
$NaNO_3$, hetacillin, $NaHCO_3$	1·48 ± 0·07***	1·12 ± 0·08***	0·62 ± 0·05***	0·51 ± 0·06*	0·49 ± 0·05***	13·09 ± 2·24***

GIT = Gastro-intestinal tract ND = Not detected

†The concentrations of the treatment chemicals in the diet were $NaNO_3$, 0·5%, $NaHCO_3$, 0·5%, $CaCO_3$, 0·5%, Lactobacillus preparation, 2% and hetacillin, 0·05% throughout the experiment.

Values are means ± SEM for five determinations; those that differ significantly (Student's *t* test) from the corresponding values for the nitrate only treatment are marked with asterisks (*$P < 0·05$; **$P < 0·01$; ***$P < 0·005$).

tents of rats treated concurrently with sodium nitrate and Lactobacillus preparation or hetacillin with or without sodium bicarbonate are given in Table 5. A significant elevation of nitrate reductase activity was observed in the stomach and upper small intestine corresponding to the accumulation of nitrite in these segments (Tables 2 & 3).

Effects of antibiotics on the induction of nitrate reductase in E. coli *and isolated rat gastric microflora* in vitro

Tables 1 and 5 show that the presence of 0·05% sodium nitrate in the incubating medium increases the activity of nitrate reductase in the GIT contents several fold (c. ten-fold in stomach contents and two to ten-fold in the small and large intestine contents respectively). Since different antibiotics were found to exert different effects on the activity of nitrate reductase *in vivo* (Table 4), we also investigated the effects of these antibiotics on the activity of this enzyme induced by $NaNO_3$ *in vitro*.

The effects of 14 antibiotics on the induction of nitrate reductase in *E. coli* and gastric microflora are summarized in Table 6. In the nutrient-free medium containing 0·85% sodium chloride plus 0·05% sodium nitrate, no microbial multiplication would be expected and the induction of the enzyme was inhibited by most antibiotics tested. On the other hand, in the synthetic medium containing nutrients the microbes were able to multiply and grow. Under these conditions, the induction of nitrate reductase in *E. coli* was inhibited by all the antibiotics tested; while the induction of the enzyme in gastric microflora was inhibited by four antibiotics and enhanced by ten antibiotics including hetacillin, minomycin and thiamphenicol which were shown to potentiate the elevation of nitrite content in the stomach of rats treated with sodium nitrate and sodium bicarbonate (Table 4). The sensitivities of *E. coli* and gastric microflora to various antibiotics were quite different as demonstrated in their extent of growth (Table 6).

DISCUSSION

The results demonstrate that nitrate intake, microbial population and gastric pH are important factors affecting the concentration of nitrite in the stomach of rats. In order to obtain some information on the amounts of nitrate ingested by an average person, a series of studies has been carried out in this laboratory to evaluate the contribution of various sources of nitrate and nitrite to the Chinese diet (Lin, 1978; Lin & Lue, 1979). It is estimated that the majority of nitrate in the average Chinese diet comes from vegetables and cured meat. A similar estimate has been reported for the average US inhabitant; White (1975) estimated that four-fifths of the nitrate intake in the USA was from vegetables, and less than one-sixth from cured meat; two-thirds of the nitrite entering the average stomach originates from saliva and slightly less than one-third comes from cured meats.

The effect of nitrate intake on the formation of salivary nitrite has been studied in man using celery juice as the source of nitrate, the extent of nitrite formation being related to the quantity of nitrate and the oral microflora (Tannenbaum *et al.* 1976). Tannenbaum *et*

Table 4. *Effects of antibiotics and sodium bicarbonate on the nitrite formation in the stomach of rats sodium nitrate*

Treatment†	Stomach nitrite (μg/g of GIT tissue and contents combined)
Control, no treatment	ND
$NaNO_3$, $NaHCO_3$	0·81 ± 0·09
$NaNO_3$, $NaHCO_3$, Hetacillin, 0·05%	2·02 ± 0·12***
$NaNO_3$, $NaHCO_3$, Oxytetracycline, 0·05%	0·65 ± 0·10
$NaNO_3$, $NaHCO_3$, Minomycin, 0·02%	1·85 ± 0·09***
$NaNO_3$, $NaHCO_3$, Streptomycin, 0·05%	1·13 ± 0·39
$NaNO_3$, $NaHCO_3$, Chloramphenicol, 0·1%	0·96 ± 0·17
$NaNO_3$, $NaHCO_3$, Amikacin, 0·05%	0·05 ± 0·01***
$NaNO_3$, $NaHCO_3$, Thiamphenicol, 0·1%	1·63 ± 0·28*

GIT = Gastro-intestinal tract ND = Not detected

†The concentrations of $NaNO_3$ and $NaHCO_3$ in the diet were both 0·5% throughout.

Values are means ± SEM for five determinations; those that differ significantly (Student's *t* test) from the $NaNO_3$ and $NaHCO_3$ only treatment are marked with asterisks (*$P < 0·05$; ***$P < 0·005$).

al. (1974) consistently found nitrite in human saliva at levels of 6–10 ppm; much of it was formed by microbial reduction of nitrate naturally present in saliva. In the present study, the Lactobacillus preparation exerted a profound enhancing effect on the level of nitrite in the stomach and small intestine of rats fed sodium nitrate (Table 2) and this effect was potentiated by some antibiotics (Tables 3 and 4). The activity of nitrate reductase in various gastro-intestinal flora has been demonstrated (Lin, Lue, Lai & Lin, 1978). It is well established that the different populations of gastro-intestinal flora can be dramatically modified by the administration of antibiotics and other chemotherapeutic agents (Gorbach, 1971). These anti-microbial agents may suppress the multiplication of certain nitrate-reductase-negative strains and allow some nitrate-reductase-positive strains to grow or *vice versa*.

Jejunal bacterial concentrations are elevated in 50 to 100% of patients with primary achlorhydria or with induced hypochlorhydria resulting from surgical procedures (Draser *et al.* 1979; Gorbach, 1971; Gray & Shiner, 1967). A normally functioning stomach with adequate acid may inhibit bacterial overgrowth in the duodenum (Gorbach & Tabagcholi, 1969). On the other hand, hypochlorhydria associated with even a small duodenal diverticulum can result in massive bacterial overgrowth. The administration of antacid such as sodium bicarbonate will keep intragastric pH above 4 (Price & Sanderson, 1956) and result in massive microbial overgrowth and nitrite formation in the stomach or duodenum as demonstrated in the present animal experiments (Tables 3 & 4). These findings raise the possibility that combined administration of antacids with Lactobacillus preparation and/or antibiotics is hazardous. Further study of this problem in human subjects is clearly needed.

Nitrite derived from the dietary intake of nitrates, as well as nitrite ingested with cured meats, can participate in the *in vivo* formation of nitrosamines, but nitrite formed endogenously in the intestine may be more significant in nitrosamine formation *in vivo* (Ember, 1980). Several of the common tumours in man have been produced in an animal model in

Table 5. *Effects of sodium nitrate, sodium bicarbonate, Lactobacillus and hetacillin on the activity of microbial nitrate reductase in the contents of the rat gastro-intestinal tract*

Treatment	Nitrate reductase activity (μg nitrite/unit absorbance at 575 nm/min)†				
		Small intestine		Large intestine	
	Stomach	Upper	Lower	Upper	Lower
Control, no treatment	0·72	0·49	0·43	0·67	0·47
$NaNO_3$	0·92	0·79	0·66	0·78	0·42
$NaNO_3$, Lactobacillus	1·14	0·83	0·13	0·65	0·32
$NaNO_3$, hetacillin	1·43	0·94	0·76	0·61	0·48
$NaNO_3$, Lactobacillus, $NaHCO_3$	1·89	0·84	0·37	0·75	0·44
$NaNO_3$, hetacillin, $NaHCO_3$	2·12	1·68	0·50	0·80	0·64

*The concentrations of the treatment chemicals in the diet were $NaNO_3$, 0·5%, $NaHCO_3$, 0·5%, Lactobacillus preparation, 2% and hetacillin 0·05% throughout.

†The GIT contents were isolated aseptically from each segment and transferred to the synthetic medium containing 0·05% sodium nitrate and incubated at 37°C for 2 days. The microflora were centrifuged, washed twice with 0·9% sodium chloride and assayed for nitrate reductase. One unit of absorbance at 575 nm is equivalent to approximately 10^7 cells/ml.

Table 6. *Effects of antibiotics on the induction of nitrate reductase with sodium nitrate in* E. coli *and rat gastric microflora in 0·85% sodium chloride and synthetic medium*

Antibiotic	Concentration	Nitrate reductase (μg nitrite/unit absorbance at 575 nm/min) In 0·85% NaCl		In synthetic medium		Growth (% of control)* in synthetic medium	
		E. coli	Gastric microflora	*E. coli*	Gastric microflora	*E. coli*	Gastric microflora
Control	—	0·32	0·10	2·13	1·05	100	100
Hetacillin	1 μg/ml	0·08	0·07	1·94	1·50	60	146
Penicillin G	1 U/ml	0·07	0·03	1·07	0·90	102	79
Cephalexin monohydrate	20 μg/ml	0·20	0·005	1·59	1·97	56	23
Oxytetracycline HCl	0·25 μg/ml	0·06	0·005	1·31	1·67	82	116
Minomycin	0·1 μg/ml	0·05	0·07	1·83	1·36	63	77
Tetracycline HCl	0·25 μg/ml	0·11	0	1·26	1·60	68	75
Kanamycin sulphate	5 μg/ml	0·32	0·04	1·42	0·80	68	19
Streptomycin sulphate	1 μg/ml	0·23	0·03	0	1·03	17	18
Chloramphenicol	50 μg/ml	0	0	0	0·38	0·2	25
Thiamphenicol	0·25 μg/ml	0·01	0·01	0·58	2·02	85	81
Erythromycin estolate	1 μg/ml	0	0·07	0·69	1·34	115	88
Colistin sulphate	3 U/ml	0·03	0	0·83	1·70	107	63
Clindamycin HCl	0·6 μg/ml	0·17	0·06	0·55	1·22	112	117
Amikacin	0·1 μg/ml	0·03	0	0·38	0·75	109	86

*The microbial growth of the control group was taken as 100%. (The equivalent absorbance at 575 nm was 0·65 for *E. coli* and 0·56 for gastric microflora.)

which a secondary or tertiary amino compound has been fed together with nitrite (Greenblatt, Mirvish & So, 1971; Sander & Bürkle, 1969; Shank & Newberne, 1972). These include tumours of the lung, liver, nervous system, nasal cavity and oesophagus. Therefore, it is possible that some tumours in man arise by the ingestion of one or more secondary or tertiary amines, which react with nitrite in food or saliva to form *N*-nitroso derivatives (Lijinsky, 1977; Lin & Lai, 1980; Pelayo, 1975).

There are many reports dealing with the sources of exogenous *N*-nitroso compounds (Hwang & Rosen, 1976; Lijinsky & Epstein, 1970). Recent studies have shown that the majority of *N*-nitroso compounds in the body come from *in vivo* conversion (Lijinsky, 1976). Many scientists believe that this endogenous pool of *N*-nitroso compounds may prove to be a more important exposure route than exogenous exposure with the possible exception of higher level industrial exposures (Ember, 1980). Our experimental data demonstrate that much more nitrite is formed in the GITs of rats fed nitrate than in those of control animals which are virtually free from nitrite in their GIT (Tables 2 & 3). Combined treatments with antacids, Lactobacillus preparation, and antibiotics increased the conversion of nitrate to nitrite two- to three-fold in the stomach and intestine (Tables 2 & 3). This conversion is significant although its efficiency is low. The highest yields of nitrite in the GIT of treated animals were found to be 3–5 ppm. Since the carcinogenic *N*-nitroso compounds such as dimethylnitrosamine and diethylnitrosamine have been shown to be active at ppm levels (Lijinsky, 1977; Magee & Barnes, 1967), the formation of nitrite in the GIT at ppm levels should not be overlooked. We suggest that increasing efforts should be made to elucidate the possible role of the gastro-intestinal flora and dietary nitrate in the process of environmental carcinogenesis.

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EFFETS DES DERIVES NITROIMIDAZOLES ET NITROFURAZONES SUR LA PEROXIDATION LIPIDIQUE DES MICROSOMES HEPATIQUES

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Résumé—Les rapports possibles entre les dérivés nitrés (nitroimidazoles, nitrofurazones) et la dégradation peroxydative des lipides microsomaux, ainsi que quelques systèmes enzymatiques microsomaux dans le foie du rat ont été étudiés. Quand la reaction aérobique a été précédée par une préincubation anaérobique, la formation de la malonaldéhyde et des diènes conjugués ainsi que les niveaux du cytochrome *P*-450 et l'activité de la NADPH–cytochrome *c* réductase ont été reduits sous l'action des nitroimidazoles et des nitrofurazones. Toutefois, l'activité de la *N*-déméthylase n'est pas changée. L'activité des néotétrazolium réductases est très augmentée sous l'action des nitrofurazones mais elle n'est pas changée par les nitroimidazoles. Les actions possibles des nitrofurazones et des nitroimidazoles sur la peroxydation lipidique sont discutées.

Abstract—Possible relationships between nitro compounds (nitroimidazoles and nitrofurazones) and the peroxidative degradation of microsomal lipids and several microsomal enzyme systems of rat liver were studied. When the aerobic process was preceded by an anaerobic preincubation, nitroimidazoles and nitrofurazones decreased the formation of malonic dialdehyde and conjugated dienes, the levels of cytochrome *P*-450 and the activity of NADPH–cytochrome *c* reductase, but the activity of *N*-demethylase was not affected. The activity of the neotetrazolium reductases was markedly increased by the nitrofurazones but was unaffected by the nitroimidazoles. The possible actions of nitrofurazones and nitroimidazoles on lipoperoxidation are discussed.

INTRODUCTION

Certains dérivés organonitrés, comme les nitrofurazones, les nitroimidazoles ou le chloramphénicol, ont des propriétés antibactériennes ou antiparasitaires (Andreeva, Ashgikhin et Vetra, 1973; Yurchenco, Yurchenco et Piepoli, 1953) qui les font utiliser en médecine humaine et vétérinaire; par exemple, les nitrofurazones sont des anticoccidiens reconnus, le chloramphénicol est un agent de lutte contre les Salmonelles. Du fait de leur emploi en thérapeutique vétérinaire, il n'est donc pas impossible de retrouver des résidus de ces produits ou de leurs métabolites dans l'alimentation. L'utilisation de ces dérivés est cependant limitée par leurs effets secondaires sur l'organisme, comme une aplasie de la moëlle osseuse, un effet de photosensibilisation ou d'allergie (Erslev, 1953; Welch, Lewis et Kerlan, 1954).

On commence à mieux connaître le mécanisme de l'action antibactérienne et toxique des dérivés nitrés; la réduction du groupement nitré en milieu anaérobie par des bactéries intestinales ou par les microsomes hépatiques a été largement décrite (Clarke, Wardman et Goulding, 1980; Morita, Feller et Gillette, 1971; Poirier et Weisburger, 1974), et certains auteurs pensent que les produits de réduction (nitroso ou *N*-hydroxylamine) seraient à l'origine des accidents toxiques; en effet, ces produits de réduction ont une haute affinité pour le cytochrome *P*-450 (Mansuy, Gans, Chottard et Bartoli, 1977; Sakurai, Hermann, Ruf et Ullrich, 1980).

Un autre intérêt de l'étude des mécanismes de réduction des dérivés nitrés et de leurs effets physiopathologiques, tient aussi au fait que l'emploi de dérivés nitroimidazoles a été introduit en chimiothérapie anticancéreuse, comme radio-sensibilisant vis-à-vis de plusieurs systèmes de cellules tumorales hypoxiques (Eakins, Conroy, Searle *et al.* 1976; Foster, Conroy, Searle et Willson, 1976). Le misonidazole a même été proposé, en l'absence de tout traitement par irradiations, pour ses effets cytotoxiques en milieu hypoxique (Denekamp et Harris, 1975; Fowler, Adams et Denekamp, 1976).

Nous avons montré (Derache, Boigegrain et Derache, 1981) que les produits de réduction anaérobie du chloramphénicol inhibent la formation de peroxydes lipidiques, ce qui laisse supposer un effet sur les phospholipides membranaires microsomaux. Dans ces conditions, il nous est apparu intéressant de poursuivre ce travail *in vitro* en étudiant l'action de deux nitrofurazones (nitrofurantoïne, furazolidone) et de deux dérivés nitroimidazoles (misonidazole et métronidazole), sur les systèmes microsomaux de peroxydation lipidique après incubation anaérobique, notamment en étudiant le comportement du cytochrome *P*-450 et des systèmes d'hydroxylation microsomale vis-à-vis de ces dérivés nitrés, ainsi que l'apparition de malonaldéhyde, indicateur usuel, avec l'absorbance des diènes conjugués, de la peroxydation lipidique (Recknagel, 1967).

METHODES EXPERIMENTALES

Animaux. Les rats utilisés sont des rats albinos mâles de souche Wistar, d'un poids de 180 à 200 g; ils sont nourris avec un aliment U.A.R. standard (Etabl. U.A.R., Villemoisson s/Orge) et reçoivent de l'eau *ad lib.* Les animaux sont prétraités par une injection de phénobarbital (60 mg/kg de poids pendant 3 jours). Ils sont mis au jeûne 24 heures avant le sacrifice.

Matériels. La plupart des réactifs et des solvants de classe Normapur ont été fournis par la Société Prolabo, Paris. Les autres réactifs ont été obtenus à partir des sources suivantes: glucose-6-phosphate, NADP, NAD, cytochrome *c*, citricodeshydrogenase et néotétrazolium 2,2',5,5'-tetraphenyl-3—Sigma Chemical Co., St. Louis, MO, USA; nitrofurantoïne (1-[([5-nitro-2-furanyl]méthylène)amino]-2,4-imidazolinedione) et furazolidone (3-[([5-nitro-2-furanyl]méthylène)amino]-2-oxazolidinone)—Laboratoire Oberval, Lyon; métronidazole (2-méthyl-5-nitroimidazol-1-éthanol)—Laboratoire Rhone-Poulenc, Paris; misonidazole (1-(2-nitroimidazol-1-yl)-3-méthoxypropan-2-ol; Ro-07 0582)—Laboratoire Roche (Produits), Neuilly sur Seine; Tous ces dérivés ont été purifiés par recristallisation dans l'éthanol.

Préparation des microsomes de foie de rat. Les animaux ont été tué par décapitation et les foies ont été prélevés immédiatement et lavés avec une solution de KCl isotonique glacée. A partir de cette étape, toutes les opérations sont faites à une température comprise entre 0 à 4°C. Les foies lavés sont pesés, coupés et homogénéisés avec 3 volumes d'une solution ionique (saccharose, 0,25 M; Tampon Tris, 50 mM; KCl 100 mM; NaCl, 40 mM) et 30 ml de cette suspension sont centrifugés à 12000 g pendant 10 minutes à 2°C dans une centrifugeuse MSE. Le surnageant est recueilli et le culot est rejeté. Cette opération est répétée une fois. Le surnageant recueilli à partir de la deuxième centrifugation est centrifugé à 100000 g pendant 90 minutes, avec une centrifugeuse MSE Superspeed 50 à 2°C. Le surnageant de cette centrifugation est décanté et stocké à 0°C. Le culot microsomal est récupéré avec une solution phosphate (0,1 M; pH 7,6) de telle manière que 1 g de foie frais soit présent dans 1 ml de suspension finale.

Incubations in vitro. Le milieu d'incubation de base (10 ml KH_2PO_4 0,1 M; pH 7,6) contient NADP 0,86 mM, isocitrate de sodium 12 mM, chlorure de magnésium 3,5 mM et isocitricodeshydrogenase 0,8 unités; la suspension microsomale est ajoutée de manière à obtenir 2 mg de protéines microsomales dans 10 ml de milieu. Les nitroimidazoles et nitrofurazones sont dissous dans le dimethylsulfoxide de façon à donner une solution 0,1 mM dans le milieu réactionnel. Nous avons vérifié que le dimethylsulfoxide n'avait pas d'effet sur les test utilisées. Les solutions sont bullées pendant 15 minutes par un courant d'azote désoxygéné par passage dans une solution de soude 0,1 N contenant 0,5% de sulfonate d'anthraquinone et 0,5% de dithionite de sodium. La température d'incubation est de 30°C pendant tout l'expérimentation. Le milieu a été soumis à une pré-incubation anaérobique de 10 minutes suivie d'une incubation aérobique de 30 minutes. Le dosage de la malonaldéhyde et des dienes conjugués a été faite à la fin de la période aérobique; le dosage du cytochrome *P*-450 et l'évaluation des activités enzymatiques ont été faits à la fin de la pré-incubation anaérobique après avoir centrifugé le milieu réactionnel à 15000 g pendant 30 minutes à 4°C.

Détermination des protéines microsomales. Les protéines, dans la suspension microsomale homogénéisée, ont été déterminées par la méthode de Lowry, Rosebrough, Farr et Randall (1951) en utilisant la serum albumine bovine comme standard.

Détermination du cytochrome P-*450.* Le cytochrome *P*-450 a été estimé spectrophotométriquement dans la suspension microsomale, par la méthode de Omura et Sato (1964), en utilisant un spectrophotomètre couplé à un enregistreur (Beckman model 35). Les résultats sont convertis en nmol/mg de protéines en utilisant le coefficient d'extinction millimolaire de 91 mM^{-1} à 450 nm (Omura et Sato, 1964).

Détermination de la malonaldéhyde. La malonaldéhyde a été déterminée en utilisant la méthode de Ghoshal et Recknagel (1965) modifiée par Scheig et Klatskin (1969). Les dienes conjugués ont été déterminés dans l'extrait lipidique microsomal selon la méthode de Srinivasan et Recknagel (1973).

Détermination des activité enzymatiques microsomales. Les NAD– et NADP–néotétrazolium réductases on été évalués dans la suspension microsomale selon la procédure décrite par Slater et Sawyer (1969). La NADPH–cytochrome *c* réductase a été mesurée selon la méthode de Ernster, Siekevitz et Palade (1962), en utilisant le coefficient d'extinction millimolaire du cytochrome *c* reduit moins le cytochrome *c* oxydé de 18,5 mM^{-1} cm^{-1} (Margoliash, 1954). Les *N*-déméthylases ont été évaluées par addition au milieu d'incubation de 1 mM de *N*-méthylaniline et détermination de la formaldéhyde formée selon la procédure décrite par Nash (1953).

RESULTATS

L'effet des dérivés nitrés sur la dégradation peroxydative des lipides microsomaux sont rassemblés dans le Tableau 1. L'analyse de ces résultats montre une différence très significative en ce qui concerne la formation de malonaldéhyde sous l'action des dérivés nitrofurazones (nitrofurantoïne et furazolidone). L'action des dérivés nitroimidazoles se caractérise par une inhibition moins forte mais significative de la dégradation peroxydative des lipides.

Ces dérivés nitrés montrent une nette activité sur la formation des dienes conjugués, surtout en ce qui concerne le métronidazole et la furazolidone et la nitrofurantoïne.

Après incubation en milieu anaérobie, la disparition du taux de cytochrome *P*-450 devient significative pour les nitroimidazoles et les nitrofurazones (Tableau 1).

En ce qui concerne les activités enzymatiques microsomales testées (Tableau 1), nous pouvons constater que la NADPH–cytochrome *c* réductase est très sensible aux dérivés nitroimidazoles; l'action des dérivés nitrofurazones est moins nette. Nous n'avons pas observé, dans nos conditions expérimentales, de modifications de l'activité des *N*-déméthylases, vis-à-vis de la *N*-méthylaniline.

L'activité des néotétrazolium réductases NAD- et NADP-dépendantes n'est pas touchée sous l'action

Tableau 1. *Effet des dérivés nitrés sur la dégradation peroxydative des lipides microsomaux après incubation aérobique et sur la cytochrome P-450 et les activités enzymatiques microsomales après incubation anaérobique*

Paramètre	Résultats† exprimés en moyenne ± 1 SD pour six essais				
	Témoins	Misonidazole	Métronidazole	Nitrofurantoïne	Furazolidone
Malonaldéhyde (nmol/mg‡)	34,23 ± 2,39	20,94 ± 6,38*	22,62 ± 0,51***	1,63 ± 0,17***	0,64 ± 0,26***
Diènes conjugués (DO 234 nm/mg‡)	0,388 ± 0,007	0,392 ± 0,017	0,298 ± 0,050**	0,315 ± 0,050**	0,298 ± 0,035***
Cytochrome *P*-450 (nmol/mg‡)	1,75 ± 0,23	0,84 ± 0,12***	0,82 ± 0,02***	0,21 ± 0,09***	0,14 ± 0,05***
NADPH–cytochrome *c* reductase (μmol cytochrome *c* réduit/min/mg‡)	0,307 ± 0,019	0,233 ± 0,016***	0,219 ± 0,023***	0,268 ± 0,026*	0,258 ± 0,037**
N-Déméthylase (nmol de formaldehyde formée/min/mg‡)	9,50 ± 0,51	7,61 ± 1,22	9,96 ± 0,39	8,95 ± 0,60	11,81 ± 2,61
Néotétrazolium réductase (nmol de néotétrazolium réduit/min/mg‡)					
NADH-dépendante	57,31 ± 2,11	54,25 ± 2,78	59,05 ± 1,80	72,48 ± 0,94***	70,75 ± 1,28***
NADPH-dépendante	6,85 ± 0,11	6,67 ± 0,20	6,80 ± 0,09	7,48 ± 0,15**	7,15 ± 0,25**

†Signification des résultats (*t* test de Student); $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.
‡Tous les resultats sont exprimés /mg de protéines microsomales.

des dérivés nitroimidazoles. Par contre, dans nos conditions expérimentales, ces activités sont très augmentées sous l'action des nitrofurazones.

DISCUSSION

Il apparait d'aprés nos résultats, que les nitrofurazones ou leurs dérivés réduits prévient la formation de la malonaldéhyde, un effet confirmé par la diminution de diènes conjugués; le même effet se retrouve avec les nitroimidazoles mais à un moindre degré. Il est évident qu'une première explication du mécanisme de cette inhibition doit être recherchée dans un effet antioxydant exercé par les dérivés réduits; les travaux de Mason et Holtzman (1975) ont en effet montré que dans des conditions expérimentales semblables aux notres, une nitroréductase agit en milieu anaérobie avec vraisemblablement apparition de RNO_2^- (Sasame et Boyd, 1979). Les dérivés nitrés pourraient se réduire complètement en arylamines, difficiles à mettre en évidence en raison de leur forte réactivité et de leur instabilité (Feller, Morita et Gillette, 1971).

Par ailleurs nos résultats montrent une inhibition trés significative du taux du cytochrome *P*-450 pour chacun des dérivés nitrés étudiés. Les travaux de Gillette, Kamm et Sasame (1968) ont montré que le cytochrome *P*-450 peut catalyser la réduction des dérivés nitroaromatiques, mais le produit de réduction, au moins des petites molécules (nitroalkanes) pouvait réagir avec le cytochrome *P*-450 pour former des complexes plus ou moins stables (Jonsson et Lindeke, 1976; Mansuy *et al.* 1977; Mansuy Rouer, Bacot *et al.* 1978). On sait par ailleurs que le cytochrome *P*-450, ou une structure héminique, est nécessaire à la peroxydation des lipides (Baird, 1980). Ainsi le ralentissement des réactions de peroxydation que nous avons observé pourrait également trouver une explication dans une moindre disponibilité du cytochrome *P*-450.

Aucune différence significative n'a été observée en ce qui concerne l'activité des néotétrazolium réductases NADH- et NADPH-dépendantes sous l'action des dérivés nitroimidazoles. Par contre, une différence très significative est observée en ce qui concerne l'activité des nitrofurazones. Ces résultats sont à rapprocher de l'inhibition modérée de la formation de malonaldéhyde sous l'action des dérivés nitroimidazoles et, au contraire, forte sous l'action des dérivés nitrofurazones. On pourrait expliquer cette différence par un pouvoir de réduction plus important des composés furaniques réduits en milieu anaérobie, vis-à-vis du néotétrazolium. Cette réaction, enzymatique ou chimique, mérite des investigations complémentaires.

Dans nos conditions expérimentales, l'activité de la NADPH–cytochrome *c* réductase décroit de façon nette. Ceci est à rapprocher de l'inactivation du cytochrome *P*-450 rapporté plus haut. Il est connu que la NADPH–cytochrome c réductase de foie de rat, peut générer l'anion superoxyde O_2^- responsable de la peroxydation lipidique (Pederson et Aust, 1972). Dès lors, on peut rapprocher l'inhibition de la NADPH–cytochrome *c* réductase et du cytochrome *P*-450 de la peroxydation des lipides: l'inhibition de la dégradation peroxydative des lipides peut être la conséquence de l'affinité des produits de réduction des dérivés nitrés pour les structures héminiques. Il en résulterait

une diminution de la production de l'anion superoxyde et, par conséquent, une diminution de la peroxydation des lipides membranaires.

En conclusion, la toxicité des nitrofurazones et des nitroimidazoles revêt un double aspect: s'il est bien établi que les produits de réduction des dérivés nitrés exercent un effet cytotoxique en se fixant sur les groupements héminiques, nos résultats montrent également que les dérivés nitrés, réduits en anaérobiose, se comportent comme des inhibiteurs de la dégradation peroxydative des lipides membranaires. Dès lors, de nombreuses similitudes de comportement sont observées entre la toxicité des dérivés nitrés et le mécanisme d'action toxique d'herbicides du type paraquat (Bus, Aust et Gibson, 1974; Sasame et Boyd, 1979).

Des études complémentaires sont nécessaires dans le but de préciser l'existence d'un possible cycle redox des dérivés nitrés en liaison avec la peroxydation des lipides membranaires.

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SHORT PAPERS

LACK OF MUTAGENS IN DEEP-FAT-FRIED FOODS OBTAINED AT THE RETAIL LEVEL

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Abstract—The basic methylene chloride extract from 20 of 30 samples of foods fried in deep fat failed to elicit any mutagenic response that could be detected in the *Salmonella typhimurium*/mammalian microsome assay. The basic extracts of the remaining ten samples (all three chicken samples studied, two of the four potato-chip samples, one of four corn-chip samples, the sample of onion rings, two of six doughnuts, and one of three samples of french-fried potato) showed evidence of weak mutagenic activity. In these samples, amounts of the basic extract equivalent to 28·5–57 g of the original food sample were required to produce revertants at levels of 2·6–4·8 times the background level. Only two of the acidic methylene chloride extracts from the 30 samples exhibited mutagenic activity greater than 2·5 times the background reversion level, and in both cases (one corn-chip and one shrimp sample) the mutagenic response was quite weak. The basic extract of hamburgers fried in deep fat in a home-style fryer possessed higher levels of mutagenic activity (13 times the background reversion level). However, the mutagenic activity of deep-fried hamburgers is some four times lower than that of pan-fried hamburgers.

Introduction

Mutagens and/or carcinogens have been detected in cooked foods prepared under a variety of conditions. Benzo[*a*]pyrene, a well-known carcinogen, and other polycyclic aromatic hydrocarbons can be produced during the smoking or charbroiling of meat (Lijinsky & Shubik, 1964 & 1965; Masuda, Mori & Karatsune, 1966; Panalaks, 1976). The heating of proteinaceous foods at temperatures in excess of 570°F leads to the generation of amino acid pyrolysis products which are very potent mutagens (Matsumoto, Yoshida, Mizusaki & Okamoto, 1977; Nagao, Honda, Seino *et al.* 1977a,b). Mutagens can also be formed during the surface frying of ground beef at temperatures of approximately 400°F (Commoner, Vithayathil, Dolara *et al.* 1978; Dolara, Commoner, Vithayathil *et al.* 1979; Felton, Healy, Stuermer *et al.* 1981; Pariza, Ashoor, Chu & Lund, 1979b; Spingarn & Weisburger, 1979). Similarly, other foods cooked at comparable temperatures were found to contain mutagens, although often at lower levels than in surface-fried ground beef (Pariza, Ashoor & Chu, 1979a; Spingarn, Slocum & Weisburger, 1980). Mutagens have also been detected in beef extract prepared by a prolonged and mild heat process (Commoner *et al.* 1978).

Despite this growing awareness of a relationship between cooking and the generation of mutagens, the formation of mutagens during the deep-fat frying of foods has not been thoroughly investigated. Peanut oil was reported to be mutagenic but this was the result of contamination of the oil by aflatoxins (Fong, Ton, Koonanuwatchaidet & Huang, 1980). Processed deep-frying fats obtained from German commercial establishments were found to lack mutagenic activity (Scheutwinkel-Reich, Ingerowski & Stan, 1980a,b), but neither of these reports contained any information on the mutagenicity of foods that had been deep-fat fried. Since the temperatures of deep-fat frying are equivalent to those used in surface frying, the formation of mutagens in the foods was a distinct possibility. Therefore, this study was initiated to determine the mutagenicity of deep-fat-fried foods.

Experimental

Foods. Samples of foods fried in deep fat, including french-fried potatoes, onion rings, fish fillets, chicken, doughnuts, potato chips, corn chips, cherry pies and shrimps were obtained from local retail outlets. All of the foods had been fried prior to purchase and were tested without further processing, with the exception of the frozen deep-fat-fried shrimps which were thawed and heated in a conventional oven according to the directions on the label. Except for the chicken and cherry pies, the entire product was subjected to extraction and testing. With chicken, only the skin portion was used; with cherry pies, only the crust was used.

In addition to these commercially available products, raw ground beef was deep-fried in a home-style 5-quart deep-fat fryer. In this case, four ground-beef patties weighing (uncooked) approximately 110 g were fried at 410°F for 3 min in a shortening composed of beef fat and cottonseed oil with monoglyceride citrate, butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate and propylene glycol. The resultant hamburgers appeared very well done,

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with a dark brown colour, a crispy dehydrated texture and a cooked weight of approximately 52 g each.

Extraction procedure. A known quantity of the food sample (80–100 g) was homogenized in a blender with a sufficient quantity (300–800 ml) of 0·1 N-HCl to produce a liquid slurry. The homogenate was centrifuged at 5000 g for 20 min and then filtered through a thin layer of glass wool. The filtrate was saturated with NaCl to salt out proteins and was then filtered through a glass-wool plug. This acid filtrate (pH 2) was extracted three times with equal volumes of methylene chloride and the solvent extracts were combined. The aqueous phase was then made basic (to pH 12) with 10 N-NaOH and was extracted again three times with equal volumes of methylene chloride. Occasionally, an extract needed to be centrifuged at 5000 g for 10 min to break up an emulsion. Both the acidic and basic extracts were passed through anhydrous Na_2SO_4 to remove traces of water and were then evaporated to dryness *in vacuo*. The residues were dissolved in a small amount of 20% (v/v) methanol in chloroform, transferred to test tubes and then evaporated to dryness. Each residue was then dissolved in 0·25 ml dimethylsulphoxide for testing in the Ames assay (0·1 ml/assay). All reported values are based on cooked weight.

Mutagenesis testing. Assays were conducted using the bacterial mutagenesis test of Ames, McCann & Yamasaki (1975) as modified by Pariza *et al.* (1979b). The test organism was *Salmonella typhimurium* TA98 (provided by Professor B. N. Ames, University of California, Berkeley), plated at approximately $6·6 \times 10^9$ bacteria per plate. The rat-liver S-9 mix was obtained from Aroclor 1254-induced rats. Reported values are the means of four plates unless otherwise specified. Two replicate plates were assayed for each of duplicate food samples. All reported values have been corrected for background rates.

Results

Of the 30 samples of foods fried in deep fat and obtained at the retail level, only 12 samples gave any indication of the presence of mutagens capable of causing reversion of *S. typhimurium* strain TA98 (Table 1). With ten of these 12 samples, the mutagenic activity was present in the basic methylene chloride

Table 1. *Mutagenicity* of foods fried in deep fat and obtained from commercial outlets*

Product	Test concn (g-equivs/plate†)	No. of induced revertants per plate/ spontaneous level of revertants	
		Acidic extract	Basic extract
Fish fillets	28·5	2·01	2·02
	37	1·19	1·29
	39	1·19	1·96
	39	1·35	2·31
French-fried potatoes	28·5	1·82	3·02
	47	0·96	1·96
	78	2·04	1·84
Cherry pies	28·5	1·82	2·43
	37	0·95	1·47
Chicken	50	1·92	—
	36	—	3·57
	57	1·11	4·76
	37	1·19	2·62
Doughnuts	57	1·11	4·11
	37	0·43	2·70
	37	1·57	1·38
	50	1·18	1·46
	100	1·26	1·36
	150	1·04	1·80
Potato chips	57	1·88	1·69
	37	1·88	2·80
	37	1·94	2·62
	37	1·16	1·47
Corn chips	57	2·81	1·42
	37	1·57	1·13
	37	1·88	1·98
	37	2·19	3·00
Shrimp	37	0·62	1·52
	37	1·53	1·69
	37	2·69	1·19
Onion rings	57	1·31	2·65

*Determined by the *Salmonella typhimurium*/mammalian microsome assay, using *S. typhimurium* strain TA98, the background reversion rate for which ranged from 13 to 33.

†Weight of original food sample equivalent to dose of extract applied to plate.

extract, while in the other two cases, the mutagen was in the acidic methylene chloride extract. Although mutagenic activity was present in the basic extract of all the samples of chicken and of the onion rings, mutagenic activity was for the most part sporadically associated with the various types of deep-fried foods. Mutagenic activity was detected in the basic extracts of two of four potato-chip samples, all three chicken samples, one of four corn-chip samples, two of six doughnut samples, the sample of onion rings and one of three samples of french-fried potato. The acidic extracts, however, showed mutagenic activity only in the cases of one of the four corn-chip samples and one of the three shrimp samples.

The level of mutagenic activity in these 12 samples was extremely low. Between 28·5 and 57 g-equivalents (amounts of the original food sample) of the extracts was necessary to provide a minimally significant level of mutagenic activity. The criterion for significant mutagenic activity was a level sufficient to produce 2·5 times more revertants than were observed in control plates. The 12 positive samples produced revertants at levels of 2·6–4·8 times the background level. In addition, no dose-response relationship could be established for mutagenic activity in any of these types of deep-fried foods.

Ground beef fried in deep fat at 410°F for 3 min in a home-style fryer was found to possess considerably more mutagenic activity than any of the retail food samples. The basic methylene chloride extract of this ground beef yielded a corrected average of 559 revertants/plate (two samples) when 104 g-equivalents of extract were tested. An equal amount of the acidic methylene chloride extract produced a corrected average of only 17 revertants/plate.

Discussion

Foods fried in deep fat and obtained at the retail level possessed little or no mutagenic activity. This finding was in sharp contrast to the levels of mutagenic activity that are produced by other methods of cooking (Commoner *et al.* 1978; Dolara *et al.* 1979; Felton *et al.* 1981; Pariza *et al.* 1979a,b; Spingarn *et al.* 1980; Spingarn & Weisburger, 1979).

Many of the foods included in this survey were starchy (french-fried potatoes, cherry-pie crusts, doughnuts, potato chips and corn chips). Starchy foods cooked by baking, toasting or surface frying were found to possess significant although low levels of mutagenic activity (Pariza *et al.* 1979a; Spingarn *et al.* 1980). Spingarn *et al.* (1980) reported that the levels of mutagenic activity in baked, toasted or surface-fried starchy foods were an order of magnitude below those found in surface-fried hamburgers. Similarly, Pariza *et al.* (1979a) indicated that cooked starchy foods possessed a low but significant level of mutagenic activity of about 0·5–1·0 revertants/g. In contrast, deep-fat fried starchy foods possessed virtually no mutagenic activity (<0·5 revertants/g).

The most striking contrast between deep-fat frying and other cooking methods in terms of the generation of mutagenic activity comes from a comparison of the levels of mutagenic activity formed during the cooking of proteinaceous foods such as ground beef, fish, shrimp and chicken. Deep-fat frying of fish, shrimp and chicken resulted in the generation of quite low and often insignificant levels of mutagenic activity. Deep-fat frying of ground beef produced revertants at a level 13 times higher than the background level. This level of mutagenic activity, while significant, was at least four times lower than the level of mutagenic activity produced during pan-frying of ground beef. Pariza *et al.* (1979b) reported approximately 20 revertants/g for the basic methylene chloride extract of pan-fried ground beef cooked at 410°F for 10 min. Dolara *et al.* (1979) also reported approximately 20 revertants/g for the basic methylene chloride extract from ground beef cooked for 3 min in an electric frying pan at 375°F. By contrast, deep-fried ground beef produced only 5·4 revertants/g after cooking at 410°F for 3 min. Although a shorter cooking time was used for the deep-fat frying than in the pan-frying studies of Pariza *et al.* (1979b), the oil allowed better contact with the product and more rapid cooking times. Clearly, ground beef fried in deep fat contains less mutagenic activity than surface-fried ground beef.

The reasons for the lower levels of mutagenic activity associated with deep-fat frying are unclear, although the presence of antioxidants and high fat levels, the partial exclusion of oxygen and uniform cooking temperatures may be important factors. Although the frying fat was not directly assayed for mutagenicity, we are reasonably certain that the mutagen was not formed and then extracted into the frying fat. When a known amount of beef extract capable of causing a known level of mutagenic response was mixed with the ground beef prior to frying, all of the mutagenic activity was recovered in the ground beef. This finding indicates that the beef-extract mutagen which is similar to the ground-beef mutagen is soluble to only a limited extent in the frying fat.

This survey was concerned mainly with samples obtained at the retail level. In general, optimal frying conditions would be used in large-volume commercial establishments, which were the source of these samples. Different conditions might exist in lower-volume commercial establishments and in domestic kitchens. The effects that abuse conditions, such as extended frying times, high frying temperatures and prolonged use of the same batch of oil, may have on the generation of mutagens in foods fried in deep fat are unknown. Further studies will be necessary to establish the importance of these factors. However, it is clear that the most commonly available deep-fried foods possess little mutagenic hazard.

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N-NITROSODIMETHYLAMINE IN DOMESTIC BEER IN CHINA

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Abstract—Samples of domestic (Chinese) beer were analysed for *N*-nitrosamines using gas chromatography–high resolution mass spectrometry with a peak-matching technique. Of 26 samples tested, 20 (77%) were found to be contaminated with *N*-nitrosodimethylamine (NDMA). The mean content of these samples was 2·7 μg/litre, without correction for recovery of NDMA from beer (74% with a coefficient of variance of 18%). The remaining six samples contained <0·5 μg/litre, the lower limit of detection.

Introduction

N-Nitrosamines as a group are well known for their carcinogenic activity. In the past 10 years much attention has been focused on the levels of nitrosamines in cured meat and fish and other meat products. Recently some workers (Goff & Fine, 1979; Scanlan, Barbour, Hotchkiss & Libbey, 1980; Spiegelhalder, Eisenbrand & Preussmann, 1979) have analysed nitrosamines in beer. Spiegelhalder *et al.* (1979) reported that of 158 samples of beer, 111 (70%) were found to contain NDMA, the mean content being 2·7 μg/litre. Other workers have reported similar results. In view of the large amounts of beer consumed daily, the monitoring of nitrosamines in beer is important.

We have compared various MS techniques and have found high-resolution electron impact MS with peak matching to be just as useful as the recently developed MS technique using a chemical ionization source. Samples of some beers manufactured and consumed in China were monitored for nitrosamines in a sensitive gas chromatograph and the findings were then confirmed by HEI MS with peak matching. The results, reported in this paper, were similar to those described in the papers cited above.

Experimental

Test samples. Samples of bottled beer were collected in four Chinese cities (in Beijing, Changchun, Jilin and Siping) and within a week of collection were analysed by a procedure previously studied by us in detail. The 26 samples analysed represented 11 different brands.

Procedures. A mixture of 200 ml beer, 120 g sodium chloride and 100 ml distilled water was steam distilled in a 1-litre distillation flask and the distillate (about 300 ml) was collected in a 500-ml vessel containing 40 ml dichloromethane and a few pieces of ice. After addition of 80 g sodium chloride and 3 ml 25% (v/v) H_2SO_4 the distillate was extracted four times with 40 ml dichloromethane and the combined extracts were dried with 10 g anhydrous sodium sulphate and concentrated to 1 ml in a Kuderna-Danish evaporator in a water-bath at 50°C. The concentrated extract was purified on a glass column (1·5 mm ID) containing 3 g neutral alumina (70–200 mesh), 3 g basic alumina (70–200 mesh) and 2 g anhydrous sodium sulphate; both types of alumina had been calcined at 500°C for 4 hr, cooled and mixed thoroughly with 6% (w/w) of water. The concentrated extract was washed onto the column with 20 ml pentane and eluted with 15 ml ether, the pentane washings being discarded. The ether eluate was concentrated to 1·0 ml using a micro Snyder column at 40°C.

Primary screening for *N*-nitrosamines was carried out in a GC-7A Gas Chromatograph (Shimadzu Corp., Kyoto, Japan) with a flame thermionic detector and a 3 m × 2·1 mm (ID) glass column containing 1% KOH and 15% polyethylene glycol 20M on Chromosorb W AW-DMCS (80–100 mesh). The chromatograph was operated at a column temperature of 170°C, a detector and injector temperature of 200°C and a nitrogen carrier-gas flow of 40 ml/min. The operating conditions for the flame thermionic detector were 5 ml hydrogen/min, 280 ml air/min and a high-frequency generator setting of H and about 8. The ether solution was introduced into the column in a volume of 2 μl. The lower limit of detection for the nitrosamines studied was 0·01 ng, equivalent to 0·025 μg/litre. No false-negative data were obtained.

Positive results obtained by GC were confirmed by GC–MS. A JMS-D300 Gas Chromatograph–Mass Spectrometer (JEOL Ltd, Tokyo, Japan) was used, with a 2 m × 1·8 mm (ID) glass column packed as described above for the GC-7A instrument; the carrier gas was helium at a flow rate of 40 ml/min and the column temperature was 130°C; the interface was a glass jet separator with a solvent venting, maintained at 200°C, and the MS conditions were: resolution 7000, ionization-chamber temperature 180°C, voltage 70 V, ionization current 300 μA and emission current 3 mA, with the magnetic analyser set to resolve the

Abbreviations: DMA = Dimethylamine; GC = gas chromatography); HEI MS = high-resolution electron impact mass spectrometry; MS = mass spectrometry; NDMA = *N*-nitrosodimethylamine; NDEA = *N*-nitrosodiethylamine.

exact mass of NDMA (74·0480; peak matched against a perfluoroalkane standard, *m/e* 68·9952). The ether solutions were introduced into the GC–MS in 1-μl aliquots. The lower detection limit of 0·1 ng corresponded to a concentration of 0·5 μg/litre in the beer sample.

Recovery tests on these methods were carried out using 200 ml beer to which 5 μg standard NDMA had been added.

These procedures were also used to study the NDEA content of the beer samples, and the results of the GC–MS analyses of both NDMA and NDEA were compared with those obtained by low-resolution chemical-ionization MS.

Results and Discussion

Only one of the 11 brands of beer analysed was a dark beer; the others were light ales. The results are presented in Table 1. Of the 26 samples, 20 (77%) were found to contain NDMA, the mean content in these samples being 2·7 μg/litre. The highest content of NDMA was found in dark beer, the concentrations in the two samples of this type analysed being 6·5 and 6·0 μg/litre. The remaining six samples contained no detectable amounts of NDMA, indicating a content, if any, below 0·5 μg/litre. The average recovery of NDMA added to beer was 74% with a coefficient of variance of 18%. None of the data presented in Table 1 have been corrected for recovery. NDMA levels above 3·0 μg/litre were confirmed by high-resolution peak-matching MS. Results in agreement with these findings were also obtained using low-resolution chemical-ionization MS. All the 26 samples were analysed also for NDEA by high-resolution peak matching and low-resolution chemical-ionization MS. No sample was found to contain NDEA, although some of them gave positive results in the GC-flame thermionic detector analyses. The range of NDEA recoveries was 60–119%.

Table 1. *Concentrations of* N-*nitrosodimethylamine in beers from different areas of China*

Source	Sample no.	NDMA content† (μg/litre)
Beijing	1	3·5
	2	5·0
	3	3·5
	4	3·5
	5	6·0
	6	3·0
	7	3·5
	8	3·0
	9	0·5
	10	1·1
	11	1·1
	12	1·1
	13	ND
	14	1·1
	15*	6·5
	16*	6·0
Changchun	17	2·1
	18	1·1
	19	0·5
	20	0·5
Jilin	21	0·5
	22	ND
	23	ND
	24	ND
Siping	25	ND
	26	ND

ND = Not detectable (i.e. < 0·5 μg/litre)
*Dark beers, the other samples being light ales.
†Determined by gas chromatography–mass spectrometry.

The results of our limited work are essentially in agreement with those of Spiegelhalder *et al.* (1979). Goff & Fine (1979) proved that in their experiments no nitrosamines were formed during the analysis. It has been reported (Hrdlicka, Dyr & Kubickova, 1964) that beer contains a high level of DMA (200–300 μg/litre). This is formed in the barley kernels during malting and may be the source of NDMA. During the brewing of beer, NDMA could be formed from DMA if nitrosating agents, such as nitrogen oxides, are present. Recently, Maki, Tamura, Shimamura *et al.* (1980) studied the formation of NDMA during the brewing of beer and demonstrated that NDMA was formed mainly during the roasting of the green malt. When green malt is heated in a gas-fired kiln the concentration of NDMA in the roasted malt is some ten times higher than that in beer, being 2–76 μg/kg in the dry material. But when green malt is dried in an electric desiccator, the concentration of NDMA in the malt is greatly decreased (to 1 μg/kg). Nitrogen oxides which are formed during the burning of the gas may be the nitrosating agent. In China green malt is heated with the smoke from burning coal, a situation similar to that when gas burners are used. If the formation of NDMA is due mainly to the roasting of the green malt, the elimination of nitrosamine contamination from beer is possible and relatively simple.

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AUTORADIOGRAPHIC STUDY OF ORALLY ADMINISTERED DI-(2-ETHYLHEXYL) PHTHALATE IN THE MOUSE

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Abstract—Groups of three male mice were given single oral doses of di-(2-ethylhexyl) [^{14}C]phthalate (5 μCi; 6·72 mg/animal) and examined by whole-body autoradiography at intervals from 1 hr to 7 days after treatment. The radioactivity was excreted in the urine and faeces mainly in the first 24 hr and completely within 3–5 days. Although, following absorption, radioactivity was widely distributed, none was found in the central nervous system, skeleton or thymus, and activity was detected only rarely in the testes. There was no evidence of tissue storage.

Introduction

Toxicity data on di-(2-ethylhexyl) phthalate (DEHP), important as a PVC plasticizer, environmental contaminant and potential migrant from plastics packages into food, were reviewed in 1977 by Gray, Butterworth, Gaunt *et al.* in their report of a study in which rats fed 1 or 2% DEHP for 17 wk showed reductions in body-weight gain, increases in liver and kidney weights and testicular atrophy. A dietary level of 0·2% also had some effect on the liver and testis. Since that time, the biological effects of DEHP have been reviewed by Thomas, Darby, Wallin *et al.* (1978) and more recently a suggestion of hepatocarcinogenicity has arisen in connection with National Cancer Institute feeding studies in rats and mice (Douglas & Hartwell, 1981).

Several studies (Daniel & Bratt, 1974; Lake, Gangolli, Grasso & Lloyd, 1975; Tanaka, Adachi, Takahashi & Yamaha, 1975) have demonstrated the rapid excretion of DEHP by rats and an absence of tissue accumulation. About half of an oral dose appeared in the urine in these studies, and the rest in the faeces, some as a result of biliary excretion. Following iv injection of DEHP, Tanaka *et al.* (1975) found transient hepatic localization in rats, and Waddell, Marlowe, Miripol & Garvin (1977) found some concentration in the liver and kidney of mice, but again only at an early stage. Transfusion of leukaemic patients with platelet concentrates containing 18–38 mg DEHP/100 ml resulted in peak blood levels of 0·34–0·83 mg DEHP/100 ml, with a half-life of 28 min, and excretion of much of the dose in the urine (Rubin & Schiffer, 1976). A similar excretory pattern resulted from oral administration of the closely related diisooctyl phthalate to rats, dogs and pigs (Ikeda, Sapienza, Couvillion *et al.* 1978), except that the proportion excreted in the urine was relatively low (20–31%) in dogs and high (68–80%) in pigs. Further information on the distribution and excretion of orally administered DEHP was sought in this autoradiography study in the mouse.

Experimental

Materials. [Carbonyl-^{14}C]DEHP (specific activity 0·29 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks. It was a colourless liquid with a radiochemical purity >98% (by thin-layer chromatography) and an infrared spectrum identical with that of an authentic sample of unlabelled DEHP.

Animals. Male mice of the C57BR strain (body weight 10–12 g) were obtained from a specified-pathogen-free colony (Laboratory Animals Centre, Carshalton, Surrey), housed in an air-conditioned room and given food (Spratt's Laboratory Animal Diet. No. 1) and tap-water *ad lib.*

Procedure. The technique used was based on that of Ullberg (1954). Each of three replicate experiments involved a control mouse (killed without treatment) and eight mice given by gavage 6·72 mg [^{14}C]DEHP (5 μCi ^{14}C) dissolved in 0·2 ml corn oil after one night without food. After 1, 2, 4, 8 and 24 hr and 3, 5 and 7 days, one of the treated mice was killed by exposure to diethyl ether and immediately frozen in a solid carbon dioxide–acetone mixture. After storage at −20°C for at least 6 hr, to allow the acetone to evaporate, the frozen mouse was blocked in 1% aqueous carboxymethylcellulose, and sagittal sections (100 μm) were cut from the left of the animal to the midline. From each mouse, four sections were selected to display as many tissues and organs as possible. These sections were dried over silica gel at −20°C and placed in contact with X-ray film (Agfa Structurix DX5) for 3–5 days. The exposure time was the same for all nine animals in one experiment. The films were processed to demonstrate the areas of radioactivity. All radiographs and sections were examined by the same observer. The degree of radioactivity (blackening of the radiograph) of each tissue was identified by a five-point numerical scoring system.

Results

The radiographs from the three control mice showed no blackening. In the treated mice, no blackening was seen in the brain or spinal cord, in the bones (long bones, ribs, skull or vertebrate), in the thymus or in the lens of the eye (which was only

present in about 50% of the sections). Between 8 hr and 5 days after dosing some diffuse activity was apparent in the hair, involving the whole thickness of the pelage. One animal examined 24 hr after treatment showed granular deposits in the coat in the posterior region.

The contents of the stomach and small intestine showed marked evidence of radioactivity in all mice during the first 24 hr, but only a slight reaction in the more distal area of one animal was recorded on day 3. Radioactivity was present in the caecal contents at 1 hr, increased to a maximum at 2 hr and persisted for 1 day, but was found in only one animal (as readily visible blackening) on day 3. No radioactivity was detected in the colon contents or faeces at 1 hr, but activity was maximal in the colon contents at 2 hr and in the faeces at 4 hr. The decline in activity was similar to that in other parts of the gastro-intestinal tract.

In the bladder, there was a high level of activity between 1 and 24 hr and some activity in two of the three mice examined at 3 days, but none was detected in later sections. This activity consisted of a strong line on the bladder wall, possibly a deposit, and definite granules in the lumen. In the kidney, activity in the parenchyma was similar to that in many tissues of the same animal, but it was more concentrated in the renal pelvis and papillae.

Radioactivity in the testis was obvious in only one animal (killed at 4 hr) and was similar to the general tissue levels. A barely perceptible trace was detected in four other mice killed during the first 24 hr, being apparently confined to the tunica in the one killed at 2 hr. In other tissues the levels of radioactivity varied considerably between animals, even at the same examination interval.

To provide a basis for comparison of the tissue findings, the numbers of animals in which a specific tissue showed a level of activity greater than that in the blood are listed in Table 1. This breakdown shows that there was relatively high activity in the wall of the small intestine of most animals for 4 hr after treatment, while the more distal parts of the gastro-intestinal tract were involved only in isolated animals.

The tendency for activity in the kidney to be concentrated in the pelvis rather than the parenchyma was confirmed by this comparison. The levels of activity were markedly higher in the bladder and urine than in the blood in most of the animals killed up to 3 days after treatment.

Levels of radioactivity above the blood levels were seldom encountered in the livers of mice killed more than 4 hr after exposure and were never found in the testes.

Discussion

This study was designed to provide data on the distribution and fate of the radioactive component (the phthalate moiety) of ^{14}C-labelled DEHP administered orally to mice, but could throw no light on the fate of the alcohol likely to be released during metabolism, particularly in the intestine (Lake, Phillips, Linnell & Gangolli, 1977; Rowland, Cottrell & Phillips, 1977). Within that limitation, the study established that orally administered DEHP or its metabolites was widely distributed in the tissues but no penetration of the central nervous system, skeleton or thymus was evident. Similar exclusion from the central nervous system of the rat was reported by Tanaka *et al.* (1975), and no activity was found in the brain, thymus or skeleton of mice following iv injection of DEHP (Waddell *et al.* 1977). Only minimal radioactivity was found in the brain of dogs and pigs given diisooctyl [^{14}C]phthalate orally (Ikeda *et al.* 1978).

In view of the reported effects of DEHP on the testis (Gray *et al.* 1977) the absence of any concentration of radioactivity in that organ is of interest. The generally low activity found and the few animals affected (36% of those examined within 24 hr of treatment) suggests a limited presence in this tissue in keeping with the earlier distribution studies.

The pattern of activity in the gut contents confirms the excretion of much of the radioactive material in the faeces, mostly within 3 days and completely by day 5. A similar finding was reported in the rat (Lake *et al.* 1975). The persistence of marked activity in the contents of the stomach and small intestine for 24 hr and in the contents of the small and large intestine of one mouse up to day 3 was unexpected in view of the reported times of stomach emptying (1 hr) and intestinal transit (1 day) of a barium meal in another

Table 1. *Numbers of mice in which activity of specific tissues exceeded that of the blood following a single oral dose of di-(2-ethylhexyl) [^{14}C]phthalate*

Tissue	No.* with increased activity at					
	1 hr	2 hr	4 hr	8 hr	24 hr	3 days
Small-intestine wall	2/3	1/2	3/3	0/3	0/3	0/3
Caecum wall	0/2	0/1	1/3	0/1	0/3	0/3
Colon wall	0/3	0/1	1/1	0/3	0/2	0/2
Kidney	1/3	1/2	0/3	0/3	1/3	0/3
Renal pelvis	2/3	0/1	3/3	1/2	0/2	0/3
Bladder and urine	3/3	2/2	3/3	3/3	3/3	2/3
Epididymis	0/3	—	0/2	—	1/3	0/3
Liver	2/3	1/2	3/3	0/3	1/3	1/3
Skin	0/3	0/2	0/3	0/3	1/3	0/3

*No. of mice in which the activity was higher in the specified tissue than in the blood/no. of mice in which tissue could be identified and assessment of activity was not prevented by intense activity in adjacent tissue.

rodent, the rat (Pendergrass & Griffith, 1962). It is probable that the persisting activity was due to the re-ingestion of some excreted radioactivity as a result of normal grooming and possibly coprophagy. Enterohepatic circulation, which has been reported in rats (Daniel & Bratt, 1974) and mice (Waddell *et al.* 1977) may also have been a contributory factor. No direct evidence of biliary excretion was observed in the present study, but the high level of activity in the stomach and intestines may have obscured the fine detail of the liver. In no animal was a section of gall-bladder confirmed.

The activity in the intestinal wall during the first 4 hr after treatment was in keeping with absorption at this site. The similar observations in the liver may reflect DEHP metabolism there and are comparable with the transitory localization in the liver found after iv injection in the rat (Tanaka *et al.* 1975).

The pattern of activity in the renal system was compatible with the concentration of DEHP and its metabolites in the urine for excretion. The high activity in the lining of the bladder wall and the granular pattern in the lumen suggest the possible binding or absorption of the excretory products on other urinary components such as proteins.

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Review Section

THE THRESHOLD AND THE VIRTUALLY SAFE DOSE

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Suppose that tumours have been produced in laboratory animals (mice or rats) exposed to a carcinogen (via ingestion or inhalation) at various doses for a lifetime and that a clear dose-response pattern has emerged. If there is actual or potential human exposure to the carcinogen, current policy requires an estimate of the risk of cancer at the very low dose(s) of interest to man. This is done by extrapolation using a mathematical model (function) fitted to the observed data. It is assumed that the carcinogen produces a non-zero risk of cancer, however small the dose.

This fundamental assumption is not accepted by everyone. Some believe that for a given situation there is a threshold, below which there is a zero risk of cancer. Unfortunately, the experimental results from even the largest experiments do not appear to resolve this conflict. Thus the statistical analysis of the data may be carried using a mathematical model that assumes that a threshold exists or using one that assumes that no threshold exists. With a typical data set, both of these models might fit the data equally well in the statistical sense.

In risk assessment under the non-threshold hypothesis, one uses the fitted model to estimate the risk at low doses. Ultimately, the question of a socially acceptable risk arises. For the purpose of this discussion, let us assume that a one-in-a-million risk is socially acceptable. The dose corresponding to this risk is then usually called the virtually safe dose.

Now consider the fundamental question for this article. What is the relationship between the estimated VSD derived from a non-threshold mathematical model and the estimated threshold derived from a threshold model? To illustrate further, suppose one analyses a data set assuming a threshold and estimates the threshold for the animals to be 28 ppm. With a non-threshold model applied to the same data, would the VSD be 1000 times less (0·028 ppm), 100 times less (0·28 ppm), or ten times less (2·8 ppm)?

In the remainder of this article the estimated threshold, γ, and the estimated VSD are presented for each of 19 large data sets taken from the literature. [The sources of the data sets are given in Carlborg (1981).] A simple relationship between γ and VSD emerges from this exercise. The Weibull model will be used as the non-threshold model. The application of this model to these data sets has been discussed elsewhere (Carlborg, 1981). The threshold model here is

$$P = 1 - e^{-\alpha} \qquad \text{for } 0 < d < \gamma,$$

$$P = 1 - e^{-(\alpha + \beta(d - \gamma))} \qquad \text{for } \gamma < d,$$

where P is the probability of a tumour, d is the dose, γ is the threshold and α and β are other parameters. With $\gamma = 0$, the model reduces to the one-hit model. Weighted least squares was used to fit the model

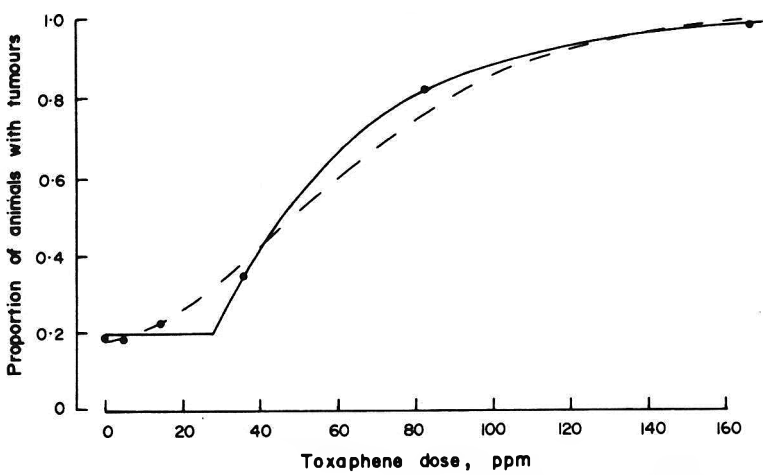

Fig. 1. The curves resulting from fitting the threshold (——) and Weibull (– – –) models to the experimental data from study 8 (liver tumours in male mice exposed to Toxaphene).

Abbreviations: VSD = Virtually safe dose.

Table 1. *Results of fitting the Weibull and threshold models to 19 data sets from long-term exposure studies in carcinogenesis*

Study no.*	Agent	Weibull model			Threshold model		
		m	*P*	VSD	γ	*P*	γ/VSD
1	2-AAF	1·49	0·56	0·0206	19·5	0·93	950
3	DDT	1·44	0·08	0·0156	33	0·06	2100
4	Dieldrin	2·10	0.99	0·00543	1·8	0·32	330
5	Dieldrin	1·41	0·32	0·000333	0·82	0·48	2500
8	Toxaphene	1·71	0·71	0·0254	27·7	0·95	1100
9	Toxaphene	1·89	0·92	0·0878	29·4	0·38	340
10	Griseofulvin	1·56	0·10	0·000260	0·25	0·74	960
11	HS	2·30	0·08	0·00171	0·14	0·29	82
12	HS	4·24	0·65	0·0213	0·26	0·39	12
14	2-AAF	1·73	0·09	0·0868	26·2	0·73	300
15	Dieldrin	1·49	0·38	0·00182	0·37	0·30	200
16	Toxaphene	2·04	0·47	0·106	31·6	0·38	300
17	Toxaphene	3·64	0·54	3·63	76·1	0·49	21
18	DMN	2·64	0·04	0·00309	0·29	0.85	94
19	2-AAF	6·46	0·15	16·6	93·5	0·18	6
23	Aflatoxin	1·58	0·64	0·00642	10·8	0·64	1700
24	Aflatoxin	1·49	0·42	0·0118	13·6	0·83	1200
25	DMN	1·43	0·24	0·00182	3·6	0·65	2000
28	ETU	3·33	0·77	6·27	218	0·90	35

2-AAF = 2-Acetylaminofluorene HS = Hydrazine sulphate
DMN = Dimethylnitrosamine ETU = Ethylene thiourea
m = The shape parameter of the Weibull model γ = The threshold
VSD = Virtually safe dose—the dose corresponding to a 10^{-6} risk
*The study numbers are those used in Carlborg (1981).

according to

$$Y = -\log_e(1 - P) = \alpha \qquad \text{for } 0 < d < \gamma$$

$$Y = -\log_e(1 - P) = \alpha + \beta(d - \gamma) \quad \text{for } \gamma < d,$$

with the weight = $(1 - P)$ (no. of animals at risk)/P.

Figure 1 illustrates the situation for Toxaphene (study 8, Table 1). There are six dose levels including the control group. Each solid dot represents an observed response. The solid curve is the best fitting threshold result. The dashed curve is the best fitting non-threshold result. From Table 1, in the Weibull model, the estimated value of the important shape parameter m is 1·71. The *P*-value for evaluating the goodness of the fit is 0·71, where a small *P*-value (like 0·05) indicates a poor fit. The estimated VSD is 0·0254 (ppm in this case). Using the threshold model, the estimated value of the threshold (γ) is 27·7 (ppm in this case), and the *P*-value is 0·95. Most important is the ratio (1100) given in the last column indicating that the VSD estimated under the Weibull model is 1100 (= 27·7/0·0254) times less than the estimated threshold.

Table 1 gives the corresponding results for all 19 data sets. Exactly the same data points were used in each data set for both models. As suggested earlier, neither the Weibull model nor the threshold model is to be preferred overall for all 19 data sets (based on the *P*-values in the fourth and seventh columns).

The eighth column of Table 2 shows a huge variation among the ratios. In Fig. 2, this ratio (logarithmic scale on the vertical axis) is plotted against the shape parameter m of the Weibull model (horizontal axis). Clearly, the variation among the ratios is largely explained by the value of m. (The dashed curve is fitted by eye.) For example, with a value of m near 2·5, the VSD is about 100 times lower than the threshold.

Figure 2 has interesting regulatory implications.

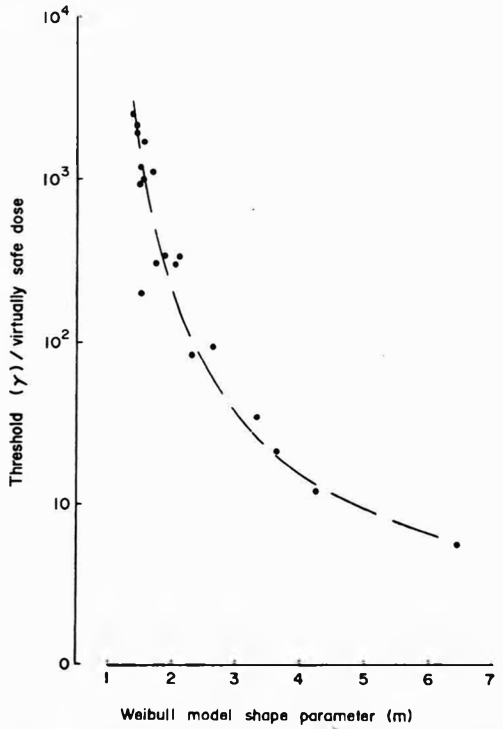

Fig. 2. The relationship between the value of the Weibull model shape parameter (m) and the ratio of the estimated threshold (threshold model) to the estimated virtually safe dose (Weibull model).

Using a non-threshold model (such as the Weibull), one might adopt the VSD as a socially tolerable level of exposure. Assuming a threshold, one might apply a 'safety factor' (say, 100) to the estimated threshold as the socially tolerable level of exposure. It is only when the dose-response curve has a steepness of $m = 2{\cdot}5$ that these two policies are equivalent. When the dose-response curve is less steep ($m < 2{\cdot}5$), then the VSD is more than 100 times less than the threshold. When the dose-response curve is more steep ($m > 2{\cdot}5$), then the VSD is less than 100 times less than the threshold.

The original investigation of the Weibull model contained 31 data sets (Carlborg, 1981). Twelve of these have been ignored here for various reasons. In four data sets (studies 2, 21, 22 and 26), the apparent threshold (γ) is zero. In four other sets (13, 29, 30 and 31), the independent variable is time, rather than the dose. In one set (study 20) the data are too meagre, and in another (study 27) the weight of the animal is the independent variable, rather than the dose. Two sets (studies 6 and 7) are composites of several smaller sets, and the appropriate definitions of the VSD and the threshold are less clear.

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PASSIVE SMOKING

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Summary—Before 1980 the argument that passive smoking was a serious health hazard was rather tenuous. It was claimed that it produced allergic reactions, impaired driving ability, reduced exercise tolerance in patients with cardiorespiratory disease and increased the risk of bronchitis and pneumonia in first-year children. However, none of these claims provided convincing evidence relevant to the normal healthy adult nonsmoker. Many studies indicate that nonsmokers are unlikely to inhale more than a very small amount of those components of tobacco smoke traditionally considered harmful. It was surprising, therefore, when a study carried out in the USA showed reduced airways function and studies from Japan and Greece showed an increased lung cancer incidence, in nonsmokers passively exposed to tobacco smoke in comparison with nonsmokers not so exposed. A review of the detail of these studies suggests that none provides conclusive evidence that passive smoking is seriously harmful, a view supported by a recent large study that was carried out in the USA and in which no significant relationship was found between passive smoking and lung cancer. More research is urgently needed, particularly to explore the influence of potentially confounding factors.

Introduction

Passive smoking is the inhalation of tobacco smoke other than by puffing on a cigarette, cigar or pipe. Study of it is relatively new, with few literature references before 1970. In this review a number of types of accusations that had been levelled against passive smoking up until 1979 are considered first. There follows a section summarizing the dosimetric aspects, understanding of which is fundamental to sensible evaluation of the epidemiological evidence, and then recent suggestions that passive smoking might be a more serious health hazard than hitherto considered likely are examined critically.

Early claims

Annoyance and irritation

That passive smoke exposure, especially under conditions of poor ventilation, can be annoying and irritating is a matter of common experience. By interviewing 250 nonallergic patients about their reaction to cigarette smoke, Speer (1968) found that 69% reported eye irritation, 32% headache, 29% nasal symptoms and 25% cough. Weber, Jermini & Grandjean (1976) found that the frequency of reported eye, nose and throat irritation increased with increasing concentrations of smoke in a sealed chamber and suggested that acrolein was the major offending substance. Subsequently, however, Hugod, Hawkins & Astrup (1978) showed that, although a gas-phase polluted atmosphere was as annoying as one polluted with whole sidestream smoke, air pollution with acrolein at three times the concentration present in sidestream smoke caused considerably less discomfort.

Allergy

The 1979 US Surgeon-General's Report (US Public Health Service, 1979) devoted a chapter to the subject of allergy and tobacco smoke. It concluded that the existence of such an allergy was not clearly established but that those with a history of allergies to other substances, expecially those with rhinitis or asthma, were more likely to report the irritating effects of tobacco smoke. Whether this was a psychological, rather than a physiological, response is open to question.

Bronchitis and pneumonia in children

Colley (1974), who has been studying respiratory symptoms in children and young adults for many years, first reported evidence of a possible effect of parental smoking in 1974. In this study a slightly increased prevalence of cough in children aged 6–14 years whose parents smoked lost its significance when parental respiratory disease was taken into account. The author noted that "there was no suggestion that exposure to the cigarette smoke generated when parents smoked had any more than a small effect upon the child's respiratory symptoms".

Later in that year Colley, Holland & Corkhill (1974) published a follow-up paper showing that in children in the first year of life, but not in the second to fifth year, prevalence of cough was significantly higher in children of parents who smoked. This excess was still significant if the analysis was restricted to those parents who did not have phlegm. Despite noting that "the association could be a result of shared genetic susceptibility to respiratory disease between parents and children, to living in the same home environment, and to cross-infection within the family"

Abbreviations: CET = Cigarette equivalent time; COHb = Carboxyhaemoglobin; FEF (25–75%) = forced mid-expiratory flow; FEF (75–85%) = forced end-expiratory flow; FEV = forced expiratory volume in 1 second; FVC = forced vital capacity; NDMA = *N*-nitrosodimethylamine; PM = particulate matter.

they concluded that "a picture has thus emerged of a serious risk to infants in the first year of life from exposure to their parents' cigarette smoke".

A further study by Leeder, Corkhill, Irving *et al.* (1976) sustained these findings, demonstrating an increased prevalence of bronchitis, pneumonia and wheeze (but not asthma) in the first year of life in children whose parents smoked. More possible explanatory factors were studied than in 1974, including whether or not a sibling had symptoms, but standardizing for these did not affect the conclusions.

Whether passive smoke in the atmosphere of the home is the cause of these infections remains open to question. The 1979 US Surgeon-General's Report (US Public Health Service, 1979) suggested that parental neglect may play a role. Also the fact that smokers are more sociable (Eysenck, 1965) could present more opportunity for the children to come into contact with infection.

Psychomotor effects

There has been some concern that relatively low levels of carbon monoxide may have an effect on psychomotor functions, especially in relation to driving a car. The literature reports a great discrepancy in the level at which blood carboxyhaemoglobin (COHb) may affect vigilance. Summarizing the literature, the 1979 US Surgeon-General's Report (US Public Health Service, 1979) concluded that effects seen at levels of COHb found in passive smoking conditions are measurable only at the threshold of stimuli perception and that effects of CO on driving performance and interactive effects of CO and alcohol are only found at higher COHb levels. A recent study by Guillerm, Radziszewski & Caille (1978), in which subjects drove a specially equipped car for 5 hours during the night, exposed either to air or CO sufficient to produce blood levels of 7 or 11% COHb, found no effect of even 11% COHb on driving precision or visual reaction time. This COHb level exceeds that achieved by all passive and indeed most active smokers.

Exercise tolerance

Aronow (1978) examined the effect of passive smoke exposure on 10 patients (two smokers, eight nonsmokers) with angina pectoris. Mean time of exercise until onset of angina in control conditions (COHb level 1·3%) was reduced by 22% after exposure to passive smoke in a ventilated room (COHb level 1·8%) and by 38% after exposure in an unventilated room (COHb level 2·3%). He also noted that the passively exposed patients had a raised heart rate and blood pressure. He attributed this to the possible absorption of nicotine, though he did not measure blood levels. The 1979 US Surgeon-General's Report (US Public Health Service, 1979) considered it unlikely that the very low levels of nicotine absorption could be responsible for these physiological changes and suggested that the response could be due to stress following anxiety or aggravation induced by the smoke-filled room.

Summary of evidence available in 1979

Taking all this evidence together, it seemed clear that, while smoking was a source of annoyance to some, although not perhaps very annoying for many, the grounds for believing it to be a health hazard were rather thin. Where adverse effects were claimed they did not apply to the normal healthy adult nonsmoker and/or were not backed by particularly solid evidence. A statement made in a leading article in the *British Medical Journal* (1978) typified the generally accepted view at the time: "For the moment most—but not all—of the pressure for people (including many smokers) to have the right to breathe smoke-free air must be based on aesthetic considerations rather than on known serious risks to health".

Dosimetry

General

A number of totally misleading statements have been made about the dose received by a passive smoker. One example is that by Repace & Lowrey (1980) who, using a theoretical model combined with measurements of cigarette smoke particulate matter (PM) in various different environments, estimated that a nonsmoking office worker exposed to moderate passive smoke inhales the equivalent, in PM terms, of five cigarettes a day while a very heavily exposed nonsmoking musician working in a night club with a chain smoker for a room-mate inhales the equivalent of 27 cigarettes a day. Study of the detail of this paper revealed that the authors had used an extremely low yielding cigarette with a PM yield of only 0·55 mg/cigarette as a basis for calculating cigarettes per day. If, more realistically, a sales weighted average cigarette with a PM yield of 17·6 mg/cigarette had been used, the appropriate exposures would have become one-sixth of a cigarette per day for the officer worker and five-sixths of a cigarette per day for the musician. Even more outlandish was the recently reported claim of Lane quoted in the national press in the UK (*Daily Mail* and *Daily Telegraph* both of 2 June 1981) that "there is now medical evidence to show that the smoke breathed in by non-smokers is 18 times higher in tar and 12 times higher in nicotine than the smoke breathed by smokers...". The source of this claim undoubtedly comes from a table published by the Laboratory of the Government Chemist (1980), which showed that the ratio of sidestream to mainstream yields was 18 for tar and 12 for nicotine when a very low tar cigarette was smoked under machine conditions. Not only had the cigarette used as a basis for comparison a tar level some 10 to 15 times less than that normally smoked, but the fundamental error of confusing sidestream yields and ambient concentrations had also been made. The concentration of sidestream smoke is measured as it leaves the burning cone of tobacco between puffs, whereas what is relevant to the passive smoker is the concentration of smoke as it reaches him after dilution by room air. Ambient concentrations vary drastically depending on the degree of room ventilation but even under conditions of poor ventilation will be very considerably less than sidestream concentrations, which a nonsmoker would only receive if he were to keep his nose right on top of the cigarette.

A number of workers have measured the concentration of smoke constituents in ambient air and in body fluids. An important study by Hugod *et al.* (1978) measured air concentrations of a number of

Table 1. *Comparison of uptake of smoke constituents in smokers and passive smokers**

Smoke constituent	Mainstream yield inhaled by smoker (mg/cigarette)	Inhaled amount in passive smoking conditions (mg/hr)†	Cigarette equivalents/hr	Cigarette equivalent time (hr)
NO	0·30	0·182	0·61	1·6
CO	18·40	9·160	0·50	2·0
Aldehyde	0·81	0·214	0·26	3·8
Acrolein	0·09	0·013	0·14	7·1
TPM	25·30	2·300	0·09	11·1
Nicotine	2·10	0·041	0·02	50·0
Cyanide	0·25	0·005	0·02	50·0

TPM = Total particulate matter

*Data from Hugod, Hawkins & Astrup (1978).

†Volunteers were exposed in a closed, unventilated room to quite severe passive smoke conditions in which the air CO concentration was kept at 20 ppm over a 3-hr period.

constituents in a closed, unventilated room in which ten volunteers were exposed to quite severe passive smoke exposure conditions in which the air CO concentration was kept at 20 ppm. Comparing the estimated inhaled amounts of each constituent with those inhaled by a smoker they calculated cigarette equivalent times (CET) in hours for seven different constituents (Table 1). It can be seen that these estimates of the time taken to inhale the equivalent of one cigarette vary widely according to the particular constituent.

Nicotine

For nicotine, Hugod *et al.* (1978) estimated it would take a passive smoker 50 hr to take in as much as would a smoker smoking one cigarette, an amount they regarded as negligible. Their results are broadly consistent with those of Hinds & First (1975) who estimated that nicotine concentrations in various public places in the USA ranged from the equivalent of one-thousandth of a filter cigarette per hour in a 'bus station waiting room up to almost one-hundredth in a cocktail lounge. Similarly Russell & Feyerabend (1975) found that nonsmokers exposed experimentally in an almost intolerably smoky room, whilst having average urinary nicotine levels almost ten times higher than nonsmokers not deliberately exposed to smoke, had urinary nicotine levels 15 times lower than average smokers.

Total particulate matter

For total particulate matter, the constituent usually considered to be related to the excess of lung cancer risk in smokers, Hugod *et al.* (1978) concluded that the CET value is "so high that the passive smoker will never inhale more than what equals ½–1 cigarette per day"—a finding consistent with the conclusions of Repace & Lowrey (1980) if adjusted so that a sensible baseline cigarette is used for comparison.

Carbon monoxide

For CO, the conclusions of Hugod *et al.* (1978) are similar to those of Russell, Cole & Brown (1973) who, working with even more extreme conditions involving twice the exposure level for CO than that used by Hugod *et al.*, found half the CET value (i.e. 1 hour). Even despite this relatively low CET value, it is most unlikely that passive smokers will achieve blood COHb levels as high as 3%, which has been claimed to decrease the threshold for intermittent claudication and angina pectoris in patients with obliterating arterial disease (Anderson, Andelman, Strauch *et al.* 1973; Aronow, Stemmer & Isbell, 1974).

N-*Nitrosodimethylamine*

N-Nitrosodimethylamine (NDMA) merits mention in the context of passive smoking because of its unusually high ratio of sidestream to mainstream smoke deliveries (Brunnemann, Fink & Moser, 1980) and of its known biological activity.

Brunnemann, Adams, Ho & Hoffmann (1978) measured the levels of NDMA in the atmosphere for several indoor locations in the USA. The highest concentration found (0·24 ng/litre) was in a bar, and the authors calculated that a nonsmoker in this situation would inhale, in 1 hour, an amount of NDMA equivalent to that inhaled by a person actively smoking 17–35 filter cigarettes. Not only was this an extremely smoky atmosphere (their equivalent figure in a bank, where smoking was permitted, being one to two filter cigarettes), but the concentration considerably exceeded that (0·07 ng/litre) found by H. Altmann (personal communication, 1981), in a small (46 m^3) unventilated conference room in which 11 people smoked 64 cigarettes in 2 hours—conditions sufficient to produce nausea in the majority of those present. The significance of these low levels of NDMA is not clear. The 1979 US Surgeon-General's Report (US Public Health Service, 1979) points out that the absorption of nitrosamine from environmental conditions is not necessarily equivalent to the absorption by smoking, while Brunnemann *et al.* (1980) have emphasized that "no epidemiological data exist linking human respiratory cancers to volatile nitrosamines".

Dosimetry—a conclusion

Hugod *et al.* (1978) concluded that "in spite of an often considerable subjective discomfort, exposing non-smokers to tobacco smoke under realistic conditions will not cause inhalation of such amounts of the components of tobacco smoke traditionally considered harmful, that a lasting, adverse health effect in otherwise healthy, grown-up individuals seems probable".

Table 2. *Vital capacities and expiratory flow rates in smokers and nonsmokers**

Sex	Group number	Smoking habits†	Percentage of predicted			
			FVC	FEV_1	FEF 25–75%	FEF 75–85%
Male	1	Nonsmokers, no smoky environment	102	103	104	120
	2	Nonsmokers, smoky environment	99	98	91	95
	3	Smokers not inhaling	96	99	92	87
	4	Smokers: 1–10 cigarettes/day	95	97	89	77
	5	Smokers: 11–39 cigarettes/day	84	86	76	68
	6	Smokers: >40 cigarettes/day	82	77	72	60
Female	1	Nonsmokers, no smoky environment	102	104	108	112
	2	Nonsmokers, smoky environment	98	99	93	85
	3	Smokers not inhaling	97	99	92	85
	4	Smokers: 1–10 cigarettes/day	96	98	89	83
	5	Smokers: 11–39 cigarettes/day	85	85	78	69
	6	Smokers: >40 cigarettes/day	78	80	72	62

*Data from White & Froeb (1980).

†Exposure to a smoky environment or consumption of cigarettes was for more than 20 yr. Group 3 includes pipe, cigar or cigarette smokers who did not inhale. Groups 4, 5 and 6 were all inhaling cigarette smokers.

Newer evidence

Effects on the small airways

In the last 2 years, some new evidence has caused a considerable amount of rethought on the passive-smoking issue. The first such evidence, published in the *New England Journal of Medicine* in March 1980, came from a study by White & Froeb (1980) of the relationship between various pulmonary function indices and passive smoking. A group of 3002 men and women who had been physiologically evaluated during a "physical fitness profile" course, and who were without a history of relevant cardiorespiratory disease, occupational exposure to dust or fumes or severe exposure to pollution at home or at work were divided into six groups according to their exposure to tobacco smoke. No significant difference was found between nonsmokers exposed to a smoky environment for more than 20 years (group 2) and nonsmokers never so exposed (group 1) as regards forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1); but nonsmokers exposed to passive smoke had statistically significant reductions in forced mid-expiratory flow (FEF 25 to 75%) and in forced end-expiratory flow (FEF 75 to 85%; Table 2).

The most surprising thing about the results of White & Froeb (1980) was that the reductions in FEF seen in group 2 were generally very similar to those seen in group 4, smokers of one to ten cigarettes per day for more than 20 years. Why should a relatively large difference (group 2 *v.* group 1) in airways dysfunction be seen as a result of an apparently relative small difference in exposure to smoke constituents when only a relatively small difference (group 4 *v.* group 2) is seen in response to what was in all probability a much larger difference in exposure?

In view of this implausible result considerable attention had to be given to the details of the study and a number of criticisms were voiced in the *New England Journal of Medicine* (Adlkofer, Scherer & Weimann, 1980; Aviado, 1980; Huber, 1980). One of the oddest things about the study was the procedure by which the sample was selected. It was stated that each candidate was classified into one of the six defined groups. Yet it is clear from Table 2 that anyone who changed smoking habits in the last 20 years does not fit into any group. Furthermore, as the authors define both groups 1 and 2 as living in a house where tobacco smoking was not permitted, what has happened to those nonsmokers, presumably in the great majority, who lived in a house where it was permitted? There are other less important omissions too (inhaling pipe and cigar smokers or cigarette smokers not allowed to smoke at work) and one might even consider it harder to find people who do fit into the groups of White & Froeb (1980) than to find ones who do not. Without an adequate explanation of this anomaly, it is difficult to have much confidence in these findings.

Lung cancer

Whilst the findings of White & Froeb (1980) relate to an index which is contentious and certainly not an accepted reliable indicator of an increased health risk, two more studies published in January 1981, by Hirayama (1981a) and by Trichopoulos, Kalandidi, Sparros & McMahon (1981), caused more attention, as both claimed that nonsmoking wives of smokers had a significantly greater risk of lung cancer than nonsmoking wives of nonsmokers.

Japanese study. Of the two studies, that by Hirayama (1981a), who followed up 91,540 Japanese nonsmoking married women aged 40 years or over in 1965 for 14 years, is the more substantial. He classified women into three groups according to the smoking habits of the husband. The results showed a highly significant trend in the risk of lung cancer with increasing smoking by the husband, with wives of heavy smokers having double the risk of wives of nonsmokers (Table 3). In contrast the wives of smokers had no significant increase in risk for emphysema, asthma, ischaemic heart disease, or cancer of the cervix or stomach.

Following critical comments by Grundmann, Müller & Winter (1981), Kornegay & Kastenbaum (1981), Macdonald (1981), Rutsch (1981) and Sterling (1981), further information on the detail of his study has been given by Hirayama (1981b, 1981c). It is useful to summarize briefly the main points raised and to consider their implications.

Table 3. *Age-occupation standardized lung cancer risk in Japanese women nonsmokers**

Smoking habit of husband	Sample size	Number of lung cancer deaths	Annual lung cancer mortality rate per 100,000	Standardized lung cancer risk ratio	Significance of trend
Nonsmokers or occasional smokers	21,895	32	8·7	1·00	
Ex-smokers or smokers of 1–19 cigarettes/day	44,184	86	14·0	1·61	$\chi^2 = 10{\cdot}88$†
Smokers of ⩾20 cigarettes/day	25,461	56	18·1	2·08	$P = 0{\cdot}001$

*Data from Hirayama (1981a).
†One degree of freedom test statistic scoring 0, 1 and 2 for the three smoking categories.

(1) It was suggested that the statistically significant χ^2 value of 10·88 shown in Table 3 might be the result of an arithmetical error as calculations by Mantel (1981), based on the unstandardized data given in the original paper, gave a χ^2 value of only 3·31 which was not significant. In fact the confusion appears to have arisen because Hirayama (1981a) had standardized for age and occupation but had not stated this clearly. This was important as the husbands who smoked were younger than those who did not. In a subsequent letter Hirayama (1981c) presented data by age and occupation which allowed one to calculate a χ^2 value of 8·70 which, while not the same as that originally quoted, was similar and significant. Surprisingly, in the same letter, Hirayama (1981c) quoted a much higher χ^2 value of 36·81 for a similar comparison (difference between nonsmoking women whose husbands have never smoked and those whose husbands have ever smoked) but this was based on a statistical error (Lee, 1981). Despite this error, it seems probable that the association found really was a significant one, though of course a significant association need not imply a significant causal effect of passive smoke exposure.
(2) For a reason that was not apparent, Hirayama (1981a) standardized for age of the husband and not for the age of the wife. However it seems unlikely this would have materially affected the findings, as, if it did cause bias, it would be expected to affect all the causes of death and not just lung cancer.
(3) Smoking habits were determined only at the beginning of the period and may have changed. Again, however, it seems unlikely that enough of these women would have taken up smoking to cause marked bias.
(4) The great majority of the lung cancers seen, 17 out of 23 in a sample, were adenocarcinomas, a type of lung cancer generally believed to be much more weakly related to smoking than squamous cell carcinoma.
(5) Evidence of trends in lung cancer rates in Japan suggest that there may be some other important cause of lung cancer which was not studied. Between 1947 and 1978, female lung cancer rates rose nine-fold and yet Hirayama's (1981a) own results show only a four-fold risk in active cigarette smokers compared with non-passively exposed nonsmokers—and relatively few women in Japan (about 15%) smoke anyway.
(6) The index of "passive" exposure used is not likely to be very accurate. Not only does the husband smoke a varying proportion of his cigarettes at home, but the wife will also be exposed to other sourses of exposure besides the husband. In principle, though, this is likely to underestimate rather than overestimate any relative risk associated with passive smoking.
(7) What is most surprising, however, is the sheer magnitude of the association. The two-fold increased risk in wives of heavier smokers is similar, in Hirayama's (1981a) study, to that of women actively smoking about five cigarettes a day, whilst it was stated that the heavy smokers smoked on average only 8·4 cigarettes a day at home and these presumably not all in the direct presence of the wife. If this is so, the study seems to be suggesting that one actively smoked cigarette is not so very different from one passively smoked one, which seems completely inconsistent with the dosimetry, especially when one realises that an active smoker probably has greater passive smoke exposure than a passively exposed nonsmoker.

Greek study. In contrast to the Japanese study, the small Greek case-control study of Trichopoulos *et al.* (1981) is relatively lightweight, being based on only 40 lung cancer cases seen in nonsmoking women. However, their results (Table 4), though having quite wide confidence limits, agree well with those of Hirayama (1981a). Taking into account a number of possible confounding factors (age, duration of marriage, occupation, schooling, residence) did not affect the general picture.

Although the trend is statistically significant, the limitations pointed out by the authors—the small number of cases, 35% of which were not cytologically confirmed, and the cases and controls being taken from different hospitals—would have meant that no great weight would have been attached to the results had they not come out at the same time as, and being supported by, those of Hirayama (1981a). It is interesting, in comparison with the Japanese study, that Trichopoulos *et al.* (1981) specifically excluded adenocarcinomas from their cases, since it was presumably implicitly assumed that this type of lung cancer was not smoking-associated.

American study. Even taken together, the Japanese and Greek studies are by no means totally convincing. Doubts as to whether such a large effect on lung cancer incidence could possibly be due to such an apparently small dose of tobacco smoke have very recently been supported by Garfinkel (1981) based on

Table 4. *Smoking habits of husbands of Greek nonsmoking women with lung cancer and of nonsmoking control women**

Smoking habit of husband		Lung cancer cases	Controls	Relative risk	Significance of trend
Nonsmokers		11	71	1·0	
Ex-smokers		6	22	1·8	
Smokers (cigarettes/day):	1–10	2	9	2·4	$\chi^2 = 6·45$
	11–20	13	32		
	21–30	4	6	3·4	$P < 0·02$
	>30	4	9		
	Total...	40	149		

*Data from Trichopoulous, Kalandidi, Sparros & MacMahon (1981).

results from the American Cancer Society's million person prospective study and the US Veterans Study. Two analyses were carried out. The first, similar to that used in the Japanese and Greek studies, showed no significant relationship between lung cancer risk and the smoking habit of the husband (Table 5). Indeed, after matching for age, occupation, education, race, urban/rural residence and absence of serious disease at the start of the study, nonsmoking women married to smokers of 20 or more cigarettes a day had an estimated risk of lung cancer virtually identical to that of non-smoking women married to non-smokers.

The second analysis found no evidence of any trend in lung cancer rates in nonsmokers over the period of either study. As death rates of smokers had increased substantially over the period, presumably mainly because of the duration of smoking effect (older smokers at the end of the period would on average have smoked for longer than similar aged smokers at the beginning of the period), one might have expected a similar rise to be seen in non-smokers, had passive smoking been a material cause of lung cancer risk in non-smokers.

Although one might argue that passive-smoking effects would be more difficult to pick up in the USA where women spend more time out of the home and marry more often [Garfinkel (1981) had no data on smoking habits of ex-husbands] than is the case in Greece or Japan, it is clear that the Garfinkel (1981) study has underlined the view that further studies are needed to explore the relationship between passive smoking and lung cancer.

Both Garfinkel (1981), and also Hammond & Selikoff (1981) in a paper reviewing findings from the Japanese and Greek studies, pointed out that it is extremely difficult to reconcile findings indicating a higher risk of lung cancer in passive smoking with results from the study by Auerbach, Garfinkel & Hammond (1979) of histological changes in bronchial epithelium taken from autopsy material. Lesions frequently seen in cigarette smokers (such as atypical nuclei and lesions similar to carcinoma *in situ*) have very rarely been found in people who have never smoked. This finding, and also the reported small doses of smoke received by nonsmokers, both suggest that passive smoking cannot play more than a very small role in the development of lung cancer, a view also reached by Lehnert (1981) who considered evidence from the USA and Japanese studies in detail. If passive smoking is not causally implicated it is of fundamental importance to try to identify the confounding or biassing factors that resulted in the higher risk of lung cancer seen in wives of smokers in the Japanese and Greek studies, but not in the American study.

Conclusion

While more research is certainly needed, there seems at present to be no convincing evidence that passive smoking results in any material risk of serious disease for the healthy nonsmoker.

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Table 5. *Lung cancer deaths amongst nonsmoking women in the USA**

Smoking habit of husband	Number of lung cancer deaths	Age standardized analysis mortality ratio	Matched group analysis mortality ratio
Nonsmoker	65	1·00	1·00
Smoker: <20 cigarettes/day	39	1·27	1·37
Smoker: ⩾20 cigarettes/day	49	1·10	1·04

*Data from Garfinkel (1981).

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REVIEWS OF RECENT PUBLICATIONS

Natural Colours for Food and Other Uses. Edited by J. N. Counsell. Applied Science Publishers Ltd, London, 1981. pp. x + 167. £13.00.

Despite the title, well over half of this book (the proceedings of a conference held in 1979) is devoted to general studies of the basic nature of colour, colour psychology and the importance of colour in food, pharmaceuticals and cosmetics. This unfortunately leaves a mere two chapters to deal specifically with natural colourants for foods. Whilst, to some extent, this disproportionate allocation of space can be ascribed to the relative paucity of data in this increasingly emotive field, the absence of even brief comments on certain important natural colourants, such as carmine, the extracts or oleoresins of turmeric and paprika, and the caramels, cannot similarly be explained away.

The initial chapter outlines in an interesting and readable way the physics of colour, its measurement and its perception by man, and the psychophysical theme is continued in the following presentation, concerned with the psychology of colour usage in foods. Reinforcing the vital and integral role of colour in man's society, three further chapters feature a discussion of its importance in foods (both to consumer and manufacturer), a review of the characteristics and biological functions of colour in nature, and a consideration of its cosmetic use by man. In the field of pharmacy, the value of colour lies particularly in preventing confusion between drug preparations, in promoting rapid identification and in marking areas of treatment.

For readers seeking the topic implied in the title the book is saved, to some degree, by two informative chapters dealing admirably with various technological aspects of some of the natural colourants used in food. The first of these chapters introduces the reader to the carotenoids—their synthesis, their chemical, physical and biological properties and their uses. Detailed discussion of the forms available commercially for use in fat- or water-based foods and their practical use in virtually all types of food is followed by a section concerned with the estimation of levels of added carotenoids in food products. The next chapter forms a similarly useful reference text for the technologist, considering in turn the occurrence, chemistry, composition, properties and applications pertaining to riboflavin, chlorophylls, betalaines and anthocyanins. The section on applications is particularly interesting, since the suitability of individual colourants for specific foods depends on a combination of the colorant characteristics (outlined in the previous sections) and the properties of the food to be coloured. This chapter is completed by a 'quick-reference' table, which clearly shows that chlorophyll and the anthocyanins (with modifications in some cases) can be adequately used in most types of food, whilst the acceptable applications of beetroot juice and the riboflavins are more restricted.

The naïve and simplistic approach of the final chapter, relating to legislation, is in line with the early sections of the book, providing no more than a very brief introduction to this complex aspect of the use of natural food colourants.

This book provides useful background reading for anyone with interests involving the colours field, since it draws together information on many aspects rarely considered together in one volume.

Fragrance and Flavour Substances. Edited by R. Croteau, D & P S Verlag, Pattensen, FRG, 1980. pp. 200. DM 54.00.

This publication contains the papers presented at the Second International Haarmann & Reimer Symposium on Fragrance and Flavor Substances held in New York in September 1979. The theme of the symposium—New Products, Processes and Aspects of Product Safety—was tackled by contributors from the USA, Germany and France.

Thirteen of the eighteen papers describe developments in the chemistry of perfumes and food flavours. Another (the first) is concerned particularly with the biosynthesis of terpenes (including *d*-camphor in sage and *l*-menthol in mint), while the last four deal with some toxicological and legislative aspects of flavours, fragrances and cosmetics.

The chemical chapters feature synthetic routes (including manufacturing processes) to flavourings and fragrances (e.g. pepper alkaloids, jasmine and sandalwood fragrances, and macrocyclic ketones) and some of their key intermediates. There are also comments on the effect structure has on odour. Thus 2-methylnonanal exhibits "a fatty, green note", whilst in contrast 3-methylnonanal gives "a lively, fresh and youthful top note".

The toxicological contributions include a brief review of the mechanism by which some fragrances cause skin sensitization, with comments on the possibilities and limitations of applying structure–activity relationships to the tentative prediction of skin-contact hazards. The final papers discuss, from various viewpoints, the FDA's approaches to the study and control of fragrances, cosmetics and food flavourings.

Although it neglects developments in analytical methods, this book provides a succinct appraisal of current fragrance and flavour research, and should interest workers in either of these areas. The absence of any sort of index, however, lessens its value as a source of specific information.

Food Chemicals Codex. 3rd Ed. Committee on Codex Specifications, Food and Nutrition Board, Division of Biological Sciences, Assembly of Life Sciences, National Research Council. National Academy Press, Washington, DC, 1981. pp. xxxi + 735. $45.00.

The specifications in the Food Chemicals Codex are now officially recognized not only by the FDA

but also, under certain conditions, by the Canadian, Australian, New Zealand and UK authorities. This latest edition shows substantial differences in format from its 9-year-old predecessor (*Cited in F.C.T.* 1973, **11**, 303), including much larger pages and two-column layout. The addition of 113 new monographs brings the total number to 776, covering over 800 substances. For the first time these include materials such as dextrose and fructose, more generally regarded as foods than as additives. Only one monograph, for aluminium sulphate solution, has been deleted because it appears to be no longer used in foods.

The monograph section previously entitled 'Specifications' has been changed to 'Requirements', and this also now includes any identification tests that previously appeared under a 'Description' section. Specifications for flavourings have been transferred to a separate tabular section, where their various physical constants may be readily compared, and a revised policy on heavy-metal limits has been adopted for certain classes of flavouring. Volatile oils prepared by distillation will need only to produce no darkening in colour when hydrogen sulphide is passed through a 1:1 acidified oil:water mixture, and no heavy metal limits are now specified for liquid aromatic flavourings prepared and/or purified by distillation. Approximately 400 infrared spectra are included as identification tests for substances for which other specific tests may be lacking.

Other changes in the present edition are the inclusion for the first time of general GMP guidelines for food chemicals, and the abandonment of a previous policy whereby the specifications for individual substances applied also to mixtures of the primary substance with additives such as anticaking agents, antioxidants and emulsifiers. As before, three supplements will be issued free of charge to holders of the Codex, and a fourth edition should be published in 5–6 years time.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Edited by F. A. Gunther. Springer-Verlag, New York, 1980. Vol. 74, pp. ix + 138, DM 49.00; Vol. 75, pp. xiv + 189, DM 54.00.

The opening contribution to the triple bill occupying the 74th volume of *Residue Reviews* is an extensive survey of the environmental occurrence and significance of molybdenum. Its role as an essential trace element for plants and animals is balanced in this text against its possible toxicity, especially to ruminants which, of all animals, seem the most sensitive both to its deficiency and to its excess. There appear to be no specific data establishing molybdenum as an essential element for man, and the evidence for its possibly beneficial effect on dental caries is conflicting. Neither is there any clear-cut evidence of human toxicity, although a high molybdenum intake associated with low calcium and high fluoride levels may accentuate osteoporosis and other pathological bone developments. This comprehensive and useful review concludes with about 180 references.

The next paper presents a detailed but rather involved analysis of current knowledge about the circulation, decomposition and stability of polychlorinated biphenyls (PCBs) in soil-plant ecosystems and interacting environments. It is particularly concerned with the possibilities and implications of waste-PCB management by land application at rates that would be both safe and economically acceptable. The volume concludes with an evaluation of the methods available for determining residue levels of fungicides used against *Botrytis cinerea* (grey-mould). The compounds used for this purpose include phthalimides, benzimidazoles, sulphonamides, oxathiins and cyclic 2,5-dichloroanilides and thus present a wide range of analytical and toxicological problems. This review covers the literature up to May 1979. It is not a particularly 'readable' chapter, but presents a lot of information on the application of classical as well as the newer chromatographic techniques to the analysis of these fungicide residues.

Volume 75 in the series is a more compact presentation, reporting a conference held in Tucson, AZ, in February 1980 on the minimization of occupational exposure to pesticides during all stages of manufacture, packaging, formulation, distribution, storage and application as well as at the field re-entry stage. The three sections of the conference dealt successively with exposure assessment, the health effects of exposure and the minimization of these effects. In addition to the 14 papers presented, the book includes the organizing committee's summary of the conclusions and recommendations that emanated from the discussion workshops held after each presentation. The result is a wide-ranging outline of the current situation, mainly from the American viewpoint, underlining problems that demand particular attention. Some of the gaps in our knowledge and many of the difficulties of deriving the maximum benefit from pesticides without creating other problems in the way of human ill-health may be only too well known, but this small volume draws them together and provides a useful survey.

Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products. Edited by R. D. Kimbrough. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980. pp xi + 406. Dfl. 195.00.

The halogenated dibenzodioxins and dibenzofurans, polychlorinated and polybrominated biphenyls and chlorinated naphthalenes and terphenyls comprise a group of structurally related compounds, some of which have enormous commercial value, particularly as flame-retardants, dielectrics, plasticizers and lubricants, whilst others are formed as unwanted contaminants in the production processes. Properties that contribute to the commercial value of the biphenyls, terphenyls and naphthalenes and also characterize the dioxin and furan by-products are insolubility in water, high boiling points, thermal stability and resistance to chemical degradation. It is these same properties, together with their potential for bioaccumulation, that have made them extremely persistent pollutants. As a whole the group exhibits a wide range of acute and chronic toxicity but the differences are more a question of degree than of lesion or effect. This book (Volume 4 in the 'Topics in Environmental Health' series) brings together an impressive collec-

tion of the known facts on all aspects of these compounds.

The text is divided into nine chapters, contributed by different authors. The first eight are organized around broad subjects, while the ninth is a group of four major contributions, together occupying a third of the book and each subdivided to consider particular incidences of human exposure. The first two chapters give a comprehensive survey of the production, properties, usage and analysis of polyhalogenated aromatic compounds and are followed by relatively brief chapters on pollution and metabolism. A contribution on specific aspects of toxicity attempts to co-ordinate the available toxicological information with a view to providing a basis for diagnosis and the prediction of acute and long-term effects likely to arise from accidental human exposure. This chapter is divided into sections that cover acute and chronic toxicology, carcinogenesis, reproduction, teratogenesis and mutagenesis, and although the subject is a difficult one because of a lack of data on some of the compounds (particularly the furans, naphthalenes and terphenyls), the author discusses the available data well and gives a clear summary after each section. The next three chapters review specific effects of these halogenated compounds, dealing in turn with the relationship between chemical structure, biochemical effects and toxicity, with hepatic porphyria, a disorder of porphyrin metabolism associated with most of the compounds considered in this book, and with the influence of these chemicals on the immune response. As already indicated, the final contributions are concerned with the effects of human exposure and discuss what has been learned from various areas of localized contamination.

The book is well presented and, to the credit of both editor and authors, each chapter is kept largely within the confines of its title and there is very little overlap. In addition, there are comprehensive lists of literature references at the end of each chapter, dated up to and including 1980. This book should be of value both to the research worker and to regulators who may be asked to give opinions on the polyhalogenated compounds, as well as to those who may have to evaluate effects of exposure.

Drug and Chemical Risks to the Fetus and Newborn. Progress in Clinical and Biological Research. Vol. 36. Edited by R. H. Schwarz & S. J. Yaffe. Alan R. Liss, Inc., New York, 1980. pp. xi + 166. $20.00.

The passage of drugs and environmental chemicals through the placenta to the foetus and the elimination of such materials in breast milk was a few decades ago considered an unlikely event. Today however, the generally held view is that most materials administered to the mother will reach the foetus or suckling infant and this imposes considerable responsibilities on government agencies, physicians, scientists and parents to ensure that these agents do not adversely affect the young developing individual. Such prevention requires knowledge of the nature of materials that produce developmental defects in the young, the manifestation of their effects, and data on their metabolism, pharmacokinetics and mechanism of action. It was with the aim of providing health professionals with the most current information about the impact of drugs and environmental chemicals upon the newborn that a symposium was held in New York in 1979, the proceedings of which have now been published as a book.

The book covers current areas of concern such as the effects of alcohol consumption, cigarette smoking and drug abuse during pregnancy and the excretion of drugs, hormones and environmental chemicals in breast milk. There is an excellent chapter by Martha Freeman and Marion Finkel outlining the responsibilities and aims of the FDA with regard to foetal protection. Regulations, testing requirements prior to marketing, surveillance programmes after marketing, labelling requirements, research, and the work of the advisory bodies are all mentioned to give a comprehensive view of the policies and programmes of the FDA. Although there is a role for the physician in detecting developmental anomalies in association with exposure to chemicals during pregnancy, the difficulty of carrying out this task is emphasized.

The lack of recognition of the foetal alcohol syndrome (FAS) until 1973 illustrates this difficulty. As with many other disorders there is no single feature of FAS that is pathognomic, thalidomide and its production of the rare deformity phocomelia being untypical of a teratogen.

One chapter of the book is devoted to a relatively new area of research, that of the paternally mediated drug effects on the foetus. Several examples of materials that affect progeny as a result of paternal exposure are given and the possible mechanisms involved are discussed.

This book is most informative. It should fulfil the Editor's aim that it should be read by students as well as by clinicians and researchers in all fields related to reproduction toxicity.

Inflammation: Mechanisms and Treatment. Edited by D. A. Willoughby & J. P. Giroud. MTP Press Ltd, Lancaster, 1980. pp. xxxiii + 873. £39.95.

This book reports the proceedings of the Fourth International Meeting on Future Trends in Inflammation organized by the European Biological Research Association and held in London in February 1980.

An opening section discusses future research into areas such as the mechanism by which macrophages recognize and react to lymphocytes participating in immune-based inflammatory responses, new developments in leucocyte chemotaxis and in the immunopathology of rheumatoid arthritis.

Several sections are concerned with recent developments in the use of anti-inflammatory and anti-rheumatic agents as well as of pharmacological mediators such as leukotriene B and benoxaprofen. A communication concerned with the anti-inflammatory activity of aspirin reviews the evidence that it acts by inhibiting fatty acid cyclo-oxygenase, thus preventing the transformation of arachidonic acid into both prostaglandins and thromboxanes, and concludes that such a simple unifying mechanism of action is an inadequate explanation of the known effects. The action of indomethacin and attempts to synthesize usable de-

rivatives that retain the efficacy of indomethacin but are less toxic form the subject of several papers, and the results of trials on one ester, tropesin, look particularly promising. Trials of other new non-steroidal anti-inflammatory drugs, including therafectin and benoxaprofen, are also discussed. Benoxaprofen seems particularly interesting, as its capacity for inhibiting cyclo-oxygenase activity is low and it appears to act by modifying macrophage function and reducing the formation of chemotaxins for these mononuclear cells. The main emphasis in these sections, however, is on mechanisms of inhibition of prostaglandin biosynthesis.

A section on experimental models of inflammation and chemotaxis discusses *inter alia* a model of macrophage accumulation using a carrageenan-induced pleurisy, an *in vitro* system for examining the interactions between neutrophils and particulate materials, *in vitro* macrophage models for detecting potential anti-arthritic drugs, and studies on the aggregation and chemotaxis of polymorphonuclear leucocytes. There are also sections on both immune and general aspects of inflammation, another is concerned with lymphocytes and the final section discusses enzymes and products of cell secretion.

The sections, in general, are not clearly differentiated, and communications concerning rheumatoid arthritis, prostaglandin synthesis, the interaction of inflammatory stimuli and actions of anti-inflammatory agents are scattered throughout the book. Consequently, whilst a great deal of information is presented, the reader would have benefited from some reorganization of the material prior to its publication, and an introductory chapter might also have been helpful.

The Cell Biology of Inflammation. Edited by G. Weissmann. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980. pp. xxii + 714. Dfl. 230.00.

The purpose of this book is to provide detailed information about cells that participate in the inflammatory process, describing both the biochemical and physiological processes involved in the cellular responses.

Two very interesting chapters discuss the role of platelets after vascular injury, not only in the coagulation process but also in other reactions. Platelets contain many intracellular inflammatory mediators including serotonin, acid hydrolases, constituents that increase vascular permeability and prostaglandins. Prostaglandins present in blood are almost entirely produced by platelets during aggregation and blood clotting. The theory is presented that the maintenance of platelets in an inactive state requires systems that keep calcium out of the cytoplasm or suppress activities that might effect the translocation of calcium.

There is a chapter each on the biological properties of neutrophils, eosinophils, and the mast cells and basophils. Of particular interest is the ability of eosinophils to limit hypersensitivity reactions caused by parasitic infections. It seems that eosinophils are able both to damage the parasite directly and to contain the allergic reactions resulting from the release of antigens by the parasite.

The chemotactic processes by which cells are recruited to inflammatory sites are discussed in another chapter. Such factors govern the adherence, orientation and locomotion of cells prior to the secretion by the cell of intracellular granule contents. Degranulation itself may have a regulatory role in chemotaxis. Degranulation at the leading edge of the cell could provide new membrane with chemo-attractant receptors, but excessive degranulation would exhaust the supply of new plasma membrane and lead to desensitization. Intracellular levels of cyclic adenosine monophosphate and cyclic guanine monophosphate, and substances that can modify their levels, such as histamine and some prostaglandins, also appear to have modulatory effects in chemotaxis. The role of calcium in various chemotactic events is discussed and the importance of biochemical events such as the methylation of carboxyl groups for modulating intracellular calcium stores and modifying membrane charge during chemotaxis is discussed. The generation of superoxide, hydrogen peroxide and hydroxyl radicals during phagocytosis is described in another chapter; these oxidative agents comprise a very effective microbicidal system against some inflammatory invading organisms.

The breakdown of connective tissues during pathological damage and the action of lysosomal enzymes in these degradation processes are discussed later in the book, and there are several chapters on the cell biology and secretory products of monocytes and macrophages. The last two chapters review the detailed cellular, biochemical and physiological changes that occur in lymphocytes during mitogen-induced blastogenesis. Lymphocyte blastogenesis *in vitro* is an important model system for studying cell differentiation, proliferation and immune function

This book describes inflammatory processes by discussing them from a cellular viewpoint. Both the interaction of ligands with receptors on the cell plasma membrane and the subsequent intracellular reactions are extensively discussed and reviewed. Above all the book illustrates the specific actions of each cell type involved in inflammatory responses, dealing with the processes by which platelets aggregate and release factors, mast cells degranulate, phagocytic cells engulf invading organisms and secrete enzymes, and lymphocytes proliferate. Overall, it presents a most comprehensive picture of complex events.

Scientific Considerations in Monitoring and Evaluating Toxicological Research. Edited by E. J. Gralla. Hemisphere Publishing Corp., Washington, DC, 1981. pp. xv + 221. $24.50.

It is a cardinal error in science to believe in the concept of fact, but it is even worse to assume that facts do not need to be established beyond reasonable doubt. The problem is that what is reasonable is subject to fashion—hence the opening statement. The basis of scientific fact is technology, and this excellent little book is really about the technical excellence needed to establish a scientific foundation for toxicology. In a dozen chapters it covers most of the elements, not in exhaustive detail (this book is not intended for the novice) but in a fashion that gives

pause for thought to those operating in other fields and that is authoritative. It is a book to be returned to again and again as problems arise, for it will always have something to offer.

Hazards in the Chemical Laboratory. 3rd Ed. Edited by L. Bretherick. Royal Society of Chemistry, London, 1981. pp. xxi + 567. £15.00.

Compared with its immediate predecessor, published in 1977, the third edition of this established handbook contains substantial revisions of the chapters on general safety considerations and procedures and an expanded 'yellow pages' section on the hazards of individual chemicals. (A chapter in the second edition, on safety in hospital biochemistry laboratories, has been omitted.)

The revisions and additions reflect the wider dissemination of toxicological and chemical-hazard information as well as the impact of legislation, including the Health and Safety at Work etc. Act 1974, new Council of Europe directives for hazard warning labels and proposed EEC regulations for radiation protection. Modified in these various ways, the book remains an essential guide for any chemical or biochemical laboratory, serving both as a reminder on safety techniques for the experienced chemist, and as a reference work for all laboratory personnel who handle chemicals.

Banbury Report 6. Product Labeling and Health Risks. Edited by L. A. Morris, M. B. Mazis & I. Barofsky. Cold Spring Harbor Laboratory, New York, 1980. pp. 325. $54.00.

In May 1980 a meeting was held at the Banbury Center to consider the effectiveness of risk labelling as a means of helping the public to avoid health hazards and as a possible alternative to the outright banning of a product or the restriction of its sale. The latter steps may be considered to be unjustified or impractical when the adverse effects are very minor or remote for the majority of consumers or, as is the case with tobacco and alcohol, when use of the product is deeply embedded in established behaviour and provides extensive employment. In such circumstances, some means of informing the consumer about possible hazards is required.

The twenty or so formal papers given at the meeting and the discussions they provoked (reported verbatim in these proceedings) present the views of producers, consumers, legislators, economists, psychologists and others. They thus demonstrate a wide range of attitudes to the use of hazard labelling, underline the many (often opposing) factors involved and, it must be said, offer little in the way of conclusions. Studies of different types of labelling and of consumer responses to health warnings on various products (including foods, drugs and cigarettes) have so far yielded somewhat conflicting results. Nevertheless it is clear from the first section of this book that a wide range of field studies is being undertaken.

The second section, on "Labeling as a communication device" presents some interesting ideas. However, some of the papers here, and to some extent elsewhere in the book, may prove heavy-going for readers who do not take readily to comments like: "The gist of the input–output matrix is that it analyzes the social influence process into a communication input that produces a persuasion output" or "this format was cognitively too complex to process efficiently, especially in a shopping environment". Brevity and clarity help in 'a proceedings environment' too! Spasmodic problems of this kind do not characterize the report of the next session, which—under the strange title "Labeling alcohol bottles with pregnancy warnings"—consists entirely of a discussion on the use of hazard labelling and other warning methods to draw attention to the foetal alcohol syndrome. Again no firm conclusions are drawn, but the report of this lively and often heated debate clearly indicates the strong feelings engendered by this question.

Contributions to the final session ranged over a variety of factors that may influence decisions on instituting hazard labelling. Not surprisingly consumer preference has emerged as a major factor in 'ban or risk-label' decisions, leading for example to the banning of minute amounts of carcinogens under the Delaney Clause and the continuing marketing of cigarettes. More unexpected is perhaps the surprise apparently felt in various quarters about the sharp consumer reactions to the proposed restrictions on saccharin—"That was the first time I'd ever seen them [consumers] opt for a little bit of cancer". While level of intelligence/education determines how well we understand the hazard warning, it is in the long run our personal inclinations that generally determine how much we heed it. Benefit-risk analysis in one form or another is an exercise for the consumer as well as the legislator.

Side Effects of Drugs Annual 5—1981. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1981. pp. xxiv + 476. Dfl. 140.00.

The fifth annual review of adverse reactions to drugs covers significant new data reported between 1 August 1979 and 31 July 1980, as well as some later reports. It begins, however, with an interesting essay which considers the levels of efficiency achieved in the adverse-reactions early-warning systems operated in the UK and elsewhere. The authors of this contribution describe the methods used to notify prescribing doctors of potential hazards indicated by evidence accumulating in the adverse reactions register, and analyse the causes and extent of the "information lag" that may impede this process.

The reactions themselves are reported in 51 reviews covering groups of related drugs. In most cases the relationship is a functional one; notable exceptions are a chapter on metals and one specifically on lithium. The chapter on miscellaneous drugs includes a few brief entries on some ancillary chemicals, including formaldehyde, propylene glycol and dimethylsulphoxide, with comment limited in some cases to a mention of the results of animal studies. More intriguing reading is perhaps provided by the previous chapter—on non-orthodox remedies ranging from acu-

puncture (and the eight groups of complications that may result from its incompetent application) to the adverse effects of various herbal remedies, doubts about ginseng and a possible hepatic reaction to an extract of the green-lipped mussel taken for rheumatoid arthritis.

The usual coding of references to indicate the type or extent of the available information has been continued. This procedure and the four indexes—of drugs, synonyms, side effects and interactions (covering Annual 4 as well as the current volume)—combine to offer ready access to the wide range of information presented in this publication.

BOOKS RECEIVED FOR REVIEW

Dietary Factors Influencing the Risk of Cancer. Vår Föda, Vol. 33, Supplement 1. Edited by A. Bruce & J.-A. Gustafsson. National Food Administration, Uppsala, Sweden, 1981. pp. 128. US $5.00.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 80. Edited by F. A. Gunther. Springer-Verlag, New York, 1981. pp. viii + 198. $31.40.

Office Workers' Survival Handbook. A Guide to Fighting Health Hazards in the Office. By M. Craig. BSSRS Publications Ltd, London, 1981. pp. 200 £3.25.

Cosmetics Ingredients—Their Safety Assessment. Edited by R. L. Elder. Pathotox Publishers, Inc., Park Forest South, IL/Raven Press, New York, 1980. pp. 172. $25.84.

Health Impacts of Polynuclear Aromatic Hydrocarbons. Edited by A. W. Pucknat. Noyes Data Corporation, Park Ridge, NJ, 1981. pp. x + 271. $39.00.

Safety Evaluation of Nitrosatable Drugs and Chemicals. Edited by G. G. Gibson & C. Ioannides. Taylor & Francis Ltd, London, 1981. pp. ix + 285. £15.00.

Fundamentals of Industrial Toxicology. By K. Anderson & R. Scott. Ann Arbor Science Publishers Inc., Ann Arbor, MI, 1981. pp. viii + 120. £8.60.

Pathology Illustrated. Edited by A. D. T. Govan, P. S. Macfarlane & R. Callander. Churchill Livingstone, Edinburgh, 1981. pp. viii + 866. £13.95.

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

Water Activity: Influences on Food Quality. Edited by L. Rockland & G. F. Stewart. Academic Press, New York, 1981. pp. xviii + 921. £33.60.

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

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Information Section

ABSTRACTS AND COMMENTS

Intestinal transformation of curcumin

Ravindranath, V. & Chandrasekhara, N. (1981). *In vitro* studies on the intestinal absorption of curcumin in rats. *Toxicology* **20,** 251.

Curcumin is the major colouring principle of the spice turmeric, in which it is found at a level of 1–5%, and there has also been considerable interest in its potential as a natural food colouring. Two earlier *in vivo* studies demonstrated only poor absorption of curcumin, up to 90% being excreted unchanged in the faeces of the rat (*Cited in F.C.T.* 1979, **17,** 413; Holder *et al. Xenobiotica* 1978, **8,** 761). However, Ravindranath & Chandrasekhara (*Toxicology* 1980, **16,** 259) found that only up to 40% of a 400-mg dose administered to rats was recovered unchanged. They also found that little more than traces of curcumin were present in the blood, liver and kidney and that there was an increase in the excretion of urinary conjugated glucuronides and sulphates. This led them to the hypothesis that curcumin is transformed during absorption and they have now carried out *in vitro* investigations designed to test this hypothesis.

Everted rat jejunal sacs filled with buffered medium were incubated with shaking and aeration for 3 hr at 37°C in a series of flasks of incubation medium (10 ml) containing 50–750 μg curcumin (added as 0·1 ml of a suspension) or 2×10^5 cpm [^{3}H]curcumin plus 'cold' curcumin to give a total of 50 μg/flask. In addition to the two experiments at each treatment level (four with the radiolabelled samples), appropriate blanks containing curcumin but no everted sacs and *vice versa* were also run. After incubation the mucosal surfaces of the sacs were washed, the serosal contents collected and the intestines were dried. Using methods sensitive enough to detect 0·5 μg curcumin, extracts of the mucosal and serosal fluids and the dried intestinal tissues were assayed for their curcumin content. The percentage of curcumin remaining in the mucosal medium was observed to increase with the amount of curcumin added to the flask up to a level of 250 μg added curcumin, above which it remained constant. At the lowest concentration (50 μg in 10 ml) nearly 80% of the added curcumin disappeared from the mucosal medium, although at higher treatment levels this was reduced to about 30%. Little of the curcumin that disappeared was accounted for (as curcumin itself) elsewhere in the system; none was found in the serosal fluid and, even at the highest concentration, only 2·5% of the added curcumin was present in the intestinal tissue.

In contrast, all of the radioactivity used in the studies with [^{3}H]curcumin was accounted for; 65% in the mucosal fluid (of which 10–20% was curcumin), 30% in the intestinal tissue and 5% in the serosal fluid. Chromatography revealed that the radioactivity in the mucosal fluid was associated with curcumin and a less polar, colourless compound. The serosal fluid contained a radioactive substance with an R_F value identical to this colourless compound but no curcumin. The results therefore support both the previous finding that curcumin itself is poorly absorbed, and the hypothesis that transformation, probably at the mucosal surface, facilitates absorption of the as-yet unidentified curcumin metabolite(s).

Alcoholic SCE levels

Butler, M. G., Sanger, W. G. & Veomett, G. E. (1981). Increased frequency of sister-chromatid exchanges in alcoholics. *Mutation Res.* **85,** 71.

The incidence of sister-chromatid exchanges (SCE) in Chinese hamster ovary cells is increased on exposure to certain chemical mutagens (Perry & Evans, *Nature, Lond.* 1975, **258,** 121). Since many carcinogens have mutagenic effects, studies on human lymphocytes have been undertaken to ascertain whether exposure to certain substances produces cytogenetic damage. Cigarette smokers (Bridges *et al. Mutation Res.* 1979, **65,** 71) and workers exposed to vinyl chloride (*Cited in F.C.T.* 1981, **20,** 142) have been found to have an increased incidence of chromosomal abnormalities, including SCE, in their lymphocytes. In the study cited above, the SCE frequencies in the peripheral blood of 11 alcoholics and of nine former alcoholics who had abstained from alcohol abuse for at least 1 yr were compared with those of ten control subjects, who had never consumed alcohol on a regular basis and admitted to imbibing less than one drink per week. None of the subjects had a history of significant illnesses, or had been exposed to drugs or X-rays for the previous 6 months. All of the controls and former alcoholics and five of the alcoholics were judged to be of adequate nutritional status.

The alcoholics had a mean duration of alcohol abuse of 17·1 yr (range 6–30 yr) and all but one of them smoked, with an average consumption of 20 cigarettes/day (range 10–45 cigarettes/day). The mean duration of alcohol abuse by the former alcoholics was 14·6 yr (mean 7–35 yr), and the average duration of recovery was 4·4 yr (range 1–10 yr). Seven of the former alcoholics smoked, with an average consumption of 24 cigarettes/day (range 7–60 cigarettes/day). Only two of the control subjects smoked ($\leqslant$20 cigarettes/day).

The mean SCE frequency of the alcoholics was 10·6 $\pm$ 0·66 and was significantly higher than that of the control subjects (8·4 $\pm$ 0·51) or of the former alcoholics (8·4 $\pm$ 0·32). Statistical analysis of the data indicated that the increase in SCE was not related to age, sex, cigarette smoking, nutritional status, duration of

alcohol abuse or type of alcoholic beverage consumed. It seems, therefore, that SCE frequency does not increase with increasing duration of alcohol consumption and that 1 yr of abstention from alcohol abuse is sufficient to allow the SCE frequency to return to that found in normal subjects.

[In an earlier, *in vitro*, study (*Cited in F.C.T.* 1979, **17**, 557) acetaldehyde, a primary metabolite of ethanol, induced cross-links in isolated DNA strands and SCE in human lymphocytes.

It is unfortunate, although probably inevitable, that the smoking habits of the control group were so different from those of the other two groups, and it is perhaps surprising that some effect of smoking did not distinguish the control subjects from the former alcoholics. However, in previous studies on smokers, considerable variations were found in SCE incidence between subjects, and Butler *et al.* (cited above) report that in some studies no induction of SCE was demonstrated in smokers.]

Alcohol and the foetus—another instalment

Kaminski, M., Franc, M., Lebouvier, M., du Mazaubrun, C. & Rumeau-Rouquette, C. (1981). Moderate alcohol use and pregnancy outcome. *Neurobehav. Toxicol. Teratol.* **3**, 173.

There is some evidence that moderate consumption of alcohol during pregnancy can affect the foetus, perhaps by causing brain damage even when outward signs of the foetal alcohol syndrome are not present (*Cited in F.C.T.* 1979, **18**, 314). A study carried out in Paris showed a relationship between moderate alcohol intake and the incidence of stillbirths and intrauterine growth retardation (Kaminski *et al. Revue epidem. santé publ.* 1976, **24**, 27). Information on alcohol consumption during pregnancy has now been collected from two surveys, both of which were designed for other purposes, and the results have been compared with those of the previous, Parisian, study.

The first, prospective, study (A) had been carried out in the main public hospitals in Paris from 1962 to 1969 and included 9236 women who were interviewed during the first trimester of pregnancy about their drinking habits. The second survey (B) was carried out in 1976 on a representative sample of 3193 births from a national study and the women were interviewed during the first week after delivery. Data for the third study (C) were collected from 1578 women in their sixth or seventh months of pregnancy who were attending one maternity hospital in Paris. The alcohol consumption per day during pregnancy was calculated in volume of wine (at 11% ethanol) for each woman in each of the studies. Moderate or heavy drinkers were defined as drinking more than 400 ml wine/day (or the equivalent in other beverages). This quantity is equivalent to about three (125 ml) glasses of wine per day or 45 ml of absolute ethanol. The percentage of women who were classed as moderate or heavy drinkers was 5·5, 6·1 and 1·6 in the three studies, respectively. Because the pregnancy outcome of the light drinkers did not differ significantly from that of the non-drinkers, these groups were combined for subsequent analysis.

In all three studies the mothers who were moderate or heavy drinkers were on average older and of higher parity and weight, had less often worked during pregnancy and were of a lower social class than the light or non-drinkers. Data from study B indicated that they also had a lower level of education and had received less antenatal care.

The significantly higher incidence of stillbirths amongst moderate and heavy drinkers found in study A was not confirmed by the other two studies, although there was an indication of a trend towards increased stillbirths in study B. However the numbers involved in studies B and C may have been too small to detect an effect. In study A the only cause of stillbirths significantly in excess was abruptio placentae. A significant increase in premature deliveries was observed in the moderate or heavy drinkers in the retrospective study (B) but not in the other two studies. This effect persisted after adjusting for age, parity, work during pregnancy, level of education, social class and degree of antenatal care.

Birth weight was significantly lower in infants of moderate or heavy drinkers in studies A and C but no such relationship was observed in study B, in spite of the higher premature delivery rate that occurred. However, in the latter study, there was a trend towards premature infants born to heavy or moderate drinkers having a lower birth weight than the premature infants of non- or light drinkers. Data from study B also showed that birth weight was significantly lower for infants of women who were moderate or heavy beer drinkers than for those with a similar consumption of wine or who were light or non-drinkers. This relationship, which was also apparent in study A, held even after allowing for social and demographic factors characterizing beer drinkers and was not explained by a higher alcohol intake.

Placental weight was significantly lower in moderate or heavy drinkers in study A, and a similar trend was observed in the other two studies, although the numbers involved were small and the differences were not significant. No relationship was found between alcohol consumption and early neonatal mortality or congenital malformations.

[No cases of foetal alcohol syndrome were reported in any of the three studies. The results are by no means conclusive, but do give some further evidence for the adverse effects of moderate drinking. A large, carefully controlled study directly aimed at ascertaining the effects of alcohol consumption on pregnancy outcome would be helpful in determining whether such effects do exist. In the meantime it would seem prudent for pregnant women to limit their alcohol consumption.]

Behavioural teratology of 2,4,5-T

Sanderson, C. A. & Rogers, L. J. (1981). 2,4,5-Trichlorophenoxyacetic acid causes behavioral effects in chickens at environmentally relevant doses. *Science, N.Y.* **211**, 593.

The Advisory Committee on Pesticides has concluded that there is no valid medical or scientific evidence that correct usage of 2,4,5-trichlorophenoxyace-

tic acid (2,4,5-T) herbicides harms humans, animals or the environment and has dismissed individual reports of miscarriages and other adverse effects in humans and animals on various grounds (*Further Review of the Safety for Use in the U.K. of the Herbicide 2,4,5-T.* London, 1980). The apparent adverse effects of 2,4,5-T may be due to the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) contaminant, for which the permissible limit in 2,4,5-T for use in the UK has been reduced to 0·01 mg/kg (*Science in Parliament* no. 164, 14 April–23 May 1980). However, in its 1980 report the Advisory Committee on Pesticides considered that recent evidence suggests that the risk to humans from TCDD may have been overestimated. However, Sjöden & Söderberg (in *Chlorinated Phenoxy Acids and Their Dioxins.* Edited by C. Ramel, p. 149. Ecological Bulletins, Sweden, 1978), showed that single injections of 100 mg 2,4,5-T/kg body weight to pregnant rats around day 8 produced long-standing behavioural and learning deficiencies in the offspring. An Australian study of the behavioural teratology of 2,4,5-T in chickens has now been reported.

2,4,5-T (containing 0·03 mg TCDD/kg) suspended in a gum tragacanth solution was administered to chicken eggs on either day 8 or day 15 of incubation at doses of 0–53 mg/kg but behavioural effects were studied only in the 0, 7, 13 and 27 mg/kg groups. Further groups received 2,4,5-T by ip injection of a suspension on day 2 after hatching at doses of 75–225 mg/kg body weight but again behavioural effects were studied only in the 0, 75 and 150 mg/kg groups. The LD_{50} on day 15 of incubation was 53 mg/kg and on day 2 after hatching was 200 mg/kg. Eggs treated on day 15 of incubation with up to 27 mg/kg showed 70% hatching. Some 5–10% of the hatched chicks showed abnormal leg development, irrespective of dose, while about 5% showed depigmentation of feathers and down. The chicks that showed no apparent morphological effects were given behavioural tests in the second week after hatching, the parameters tested being general activity, jumping frequency, ambulation and visual learning. Trends towards increased general activity, jumping frequency and ambulation were observed in chicks treated with 2,4,5-T on day 15 of incubation and day 2 after hatching, although these effects were significant only at the higher dose levels and in the case of ambulation the trend was not significant at any dose level. No effects on general activity, jumping frequency and ambulation were observed in chicks treated on day 8 of incubation. A significant slowing of visual discrimination learning was revealed in chicks treated on day 15 of incubation with 7 and 27 mg/kg but not in those treated at the other two ages.

Neuronal cell division in the chick is at its peak on day 8 of incubation and largely completed by day 15 when synaptic proliferation is occurring. Synaptic proliferation is completed before day 2 after hatching. Day 15 of incubation appears to be a stage of maximum sensitivity to the behavioural effects of 2,4,5-T and so the brain seems to be most sensitive to the effects of 2,4,5-T at the time of maximum synaptic formation. The authors point out that these adverse effects occur at lower doses than previously reported and that the 2,4,5-T used in the study contained less TCDD than that commercially available in Australia. They also consider that the evidence from metabolism and excretion studies indicates that humans are at least three times more sensitive to 2,4,5-T than are chicks and rats, and thus conclude that their results imply a risk to man from 2,4,5-T in use.

Intermittent asbestos

Davis, J. M. G., Beckett, S. T., Bolton, R. E. & Donaldson, K. (1980). The effects of intermittent high asbestos exposure (peak dose levels) on the lungs of rats. *Br. J. exp. Path.* **61,** 272.

We have previously reported a study that sought to evaluate the effects of various factors such as concentration, duration and type of exposure on the risks associated with working with asbestos (*Cited in F.C.T.* 1980, **19,** 395), and have described experimental studies investigating the influence of fibre type and size on carcinogenic potential in animals (*ibid* 1973, **11,** 338; *ibid* 1974, **12,** 591). However, all of these studies used a constant 'even' dose of the various asbestos types which is not directly comparable to the exposure of workers. In most workplaces, the levels of asbestos are generally very low but occasionally increase markedly following a breakdown in the ventilation system or during periodic mechanical servicing of apparatus. The exposure to high 'peaks' of asbestos could present its own problems in that the body's responses, particularly pulmonary clearance, could become saturated.

To study this, groups of 48 male Wistar rats were exposed 'intermittently' to 50 mg amosite or 10 mg chrysotile/m^3 air for 1 day/wk for 12 months and then kept untreated for 17 months. A chrysotile exposure level of 50 mg/m^3 was not feasible because of flocculation of the fibres in the inhalation chamber, but the levels selected for the two types of asbestos allowed a comparison with the results of previous studies involving exposure to 10 mg amosite/m^3 or 2 mg or 10 mg chrysotile/m^3 for 7 hr/day, 5 days/wk for 12 months followed by observation for 17 months. The dust clouds were generated using a modified Timbrell dust generator and were selected for size using a cyclone system to ensure a higher proportion of respirable dust in the clouds.

Groups of four animals were killed at 12 and 18 months for examination of dust deposition and retention and estimation of pulmonary fibrosis. At the end of the study (29 months) the lungs of six animals from each group were used for microscopic estimations of advanced fibrosis while the rest were examined for the presence of pulmonary neoplasms.

The results showed that the dust levels of both chrysotile or amosite in the lungs of rats after the 12-month inhalation period were similar regardless of the dosing regime. Clearance of the fibres, although slightly faster in the intermittent chrysotile group and slightly slower in the intermittent amosite group was not significantly different from the 'evenly-dosed' groups and there were no significant differences in the incidence of pulmonary neoplasms. However, levels of early peribronchial fibrosis were significantly lower for the intermittently dosed groups while in the later stages of the experiment the levels of interstitial

fibrosis were slightly (but not significantly) higher. The authors suggest that these tissue changes might indicate differences in the pattern of dust deposition in the rat lung between 'even' dosing and intermittent dosing and might warrant further investigation.

There would appear to be little evidence that intermittent high doses of asbestos present a significantly greater hazard than more constant low levels, but it is difficult to satisfactorily emulate the very large differences in levels that can be found in the industrial environment.

Inorganic fibre migration

Lee, K. P., Barras, C. E., Griffith, F. D. & Waritz, R. S. (1981). Pulmonary response and transmigration of inorganic fibers by inhalation exposure. *Am. J. Path.* **102,** 314.

Both the capacity to produce mesotheliomas *in vivo* and the *in vitro* cytotoxic activities of fibrous mineral dusts such as potassium octatitanate seem to be determined by the number of long fine fibres in the dust (*Cited in F.C.T.* 1980, **18,** 321). Potassium octatitanate fibres are similar to asbestos in their physical configuration and size distribution. It is therefore not surprising that there is now limited evidence that potassium octatitanate produces mesothelioma after intrapleural implantation in rats (Stanton & Layard, *National Bureau of Standards Special Publication 505, 1978*). The toxicology of potassium octatitanate itself has generated some interest because of its potential use, as a substitute for asbestos in filters, by the food industry. Although potassium octatitanate and asbestos fibres have strong physical similarities, the former are much more easily detected in tissues because of their dark colour and birefringence in polarized light. In the present study Lee *et al.* used potassium octatitanate (known commercially as Fybex) to study the fate of inhaled inorganic fibres.

Groups of 19–25 young adult male rats (Charles River-CD, Sprague–Dawley-derived), 12–18 Syrian male hamsters and 11–18 albino male guinea-pigs were exposed by inhalation to an average gravimetric concentration of 0·079 mg Fybex/litre of air in one experiment, and to concentrations of 0·039 mg/litre, 0·082 mg/litre and 0·37 mg/litre of air in another experiment; the numbers of Fybex fibres greater than 5 μm in length per litre of air were $2{\cdot}9 \times 10^6$/litre, $2{\cdot}9 \times 10^6$/litre, $13{\cdot}5 \times 10^6$/litre and $41{\cdot}8 \times 10^6$/litre respectively. In both experiments the animals were exposed for 6 hr/day, 5 days/wk for 3 months. They were killed for autopsy and comparison with controls after a further 15–24 months.

The exposed animals showed dose-related deposition of Fybex dust in the lungs and pulmonary fibrosis mainly in the respiratory bronchioles, alveolar ducts, and adjoining alveoli. Pulmonary fibrosis was most prominent in the rats. There was some evidence to suggest that inhalation of Fybex was directly related to the production of mesothelioma in hamsters. In the two lower dose groups exposed at the $2{\cdot}9 \times 10^6$/litre level, one of the 30 hamsters developed epithelial-type pleural mesothelioma whereas none of the 20 control hamsters had tumours. One of 13 hamsters at the $13{\cdot}5 \times 10^6$/litre dose level had a biphasic-type pleural mesothelioma and 1 of 16 hamsters in the high-dose group showed epithelial-type pleural mesothelioma with metastasis to the mediastinal lymph nodes. No mesotheliomas were found in the control or Fybex-treated rats or guinea-pigs.

In the lungs of each species, most short fibres (<5 μm) were phagocytosed by alveolar macrophages but long fibres (<10 μm) were phagocytosed by foreign-body giant cells. The Fybex-laden macrophages had entered the walls and lumina of the lymphatic and blood vessels in the lungs. In addition, large numbers of dust cells had reached the thoracic lymph nodes where they had also entered into the lumina of lymphatic and blood vessels. There was some migration of fibres into the liver and other organs and tissues. Giant cells loaded with long fibres were occasionally observed in the liver. The hearts of some hamsters revealed dust-cell infiltration with mesothelial hyperplasia and fibrosis. Intra- and extracellular migrating fibres were found in the gastro-intestinal tract, kidneys, pancreas, salivary gland, brain, meninges, pituitary gland, thyroid, adrenals, extraorbital gland and pineal body, without apparent effects. Since dust-cell accumulation was considerable in the lymph nodes draining the lung but minimal in the spleen and liver, it was suggested that the main route of dust-cell migration was *via* the lymphatic vessels.

The authors considered these results in relation to the migration of inhaled asbestos fibres. Some Fybex particles were found in the mucosa or submucosa of the gastro-intestinal tracts of the experimental animals, but not in the muscle layers of the gut. The authors therefore considered it unlikely that fibres of asbestos, ingested as contaminants of food or beverages, or from lung clearance mechanisms following inhalation exposure, can penetrate the gastro-intestinal tract and induce either gut cancer or peritoneal mesothelioma.

Heavy metal levels in pigeons

Hutton, M. & Goodman, G. T. (1980). Metal contamination of feral pigeons *Columba livia* from the London area: Part 1—Tissue accumulation of lead, cadmium and zinc. *Envir. Pollut.* (Ser. A) **22,** 207.

The abundance of alkyllead compounds due to high traffic densities in urban areas is a matter of continuing concern, and attention has recently been paid to the possible risks arising from the ingestion of lead-rich street dust, especially by children. Similarly, motor-vehicle tyres may be the source of elevated cadmium levels found in the air and dust of urban areas. In the study reported here, levels of lead, cadmium and zinc were determined in the tissues of feral pigeons caught in Chelsea, Mortlake or Heathrow Airport (4, 8 and 18 km from the centre of London, respectively). Control birds were obtained from rural Cambridgeshire.

Tissue lead concentrations increased progressively with proximity to the city centre. The highest levels were found in bone, and females from all of the London sites contained higher bone levels of lead than

did males, although the difference was significant only among the birds caught in Chelsea. No organic lead was found in any of the tissues examined. The cadmium content of the tissue of central London (Chelsea) pigeons was also greater than in the suburban sample (Mortlake), but birds from the outer suburban site (Heathrow) also had elevated tissue cadmium levels, possibly due to the large volume of air traffic, although the precise sources of the cadmium are not known at present. Tissue metal levels corresponded to the levels encountered in the intestinal contents—presumably the birds ingest food contaminated with roadside dust while feeding at ground level. The results of analyses for zinc gave some indication that zinc co-accumulates with lead in the bone tissue of urban pigeons, and that this association is not related to differences in dietary zinc intake. However, it is not clear whether this reflects an interaction of toxicological significance. There was also an apparent association between cadmium and zinc levels in the kidneys and livers of the urban pigeons.

The ability of the urban-dwelling feral pigeon to accumulate lead and cadmium draws attention to the potential hazard of ingesting street dust, particularly by children. The authors suggest that the bird could be used as a useful monitor of urban lead and cadmium contamination and as a model for the study of chronic lead toxicity.

More UV ink components identified as sensitizers

Björkner, B. (1981). Sensitization capacity of acrylated prepolymers in ultraviolet curing inks tested in the guinea pig. *Acta derm.-vener., Stockh.* **61,** 7.

Ultraviolet curing inks continue to make inroads into the market held by conventionally cured printing inks. Suspicions of a new technology have meant that reassurances on safety-in-use have been sought although the toxicological hazards associated with the use of their air-dried or heat-cured predecessors seem to have been a matter of little concern.

UV ink formulations contain acrylates that have been tested, at most, in acute oral toxicity and skin-irritation studies. Some early reports indicated that a number of the lower aliphatic multifunctional acrylates, which were then favoured, might pose a sensitization hazard to the workforce of the ink manufacturer (*Cited in F.C.T.* 1979, **17,** 313). Evidence has now been generated that suggests that acrylate-tagged prepolymers with technological advantages over the first-generation simpler acrylates may possess a similar sensitizing ability.

An epoxyacrylate, chemically defined as the diacrylate ester of a bisphenol A epoxy resin (bisphenol A-epichlorohydrin in a 1:2 ratio), and bisphenol A dimethacrylate, a methacrylic monomer based on bisphenol A (bisphenol A-ethylene oxide in a 1:2 ratio) were subjected to a standard guinea-pig maximization test according to the method of Magnusson & Kligman (*J. invest. Derm.* 1969, **52,** 268). Attempts were made to sensitize guinea-pigs by intradermal injections of 5% epoxyacrylate or 10% bisphenol A dimethacrylate in liquid paraffin followed 1 wk later by topical treatment at the injection site with the appropriate neat material. When challenged 2 wk after the induction regime, 18 of the 20 animals treated with the acrylate responded positively (with erythema and/or oedema) to a 24-hr closed-patch test with 10% epoxyacrylate in acetone, and nine of these cross-reacted to bisphenol A dimethacrylate (25%, also in acetone). (These concentrations had been shown to be non-irritating in preliminary studies.) Eight of the 20 guinea-pigs similarly treated with the methacrylate became sensitized, giving a positive response to a 25% challenge solution of the methacrylate in acetone. Rather surprisingly, 14 of these same animals responded positively to a 10% epoxyacrylate challenge. (This is an inexplicable result if the two formulations were tested simultaneously, as it indicates a higher incidence of reactions to the secondary than to the primary allergen.)

Each of the groups of 20 animals was then given a booster dose of either 5% epoxyacrylate or 10% bisphenol A dimethacrylate (0·1 ml in liquid paraffin administered intradermally) and rechallenged 1 wk later. In the epoxyacrylate group, the sensitization incidence was raised to 100%; five of the animals cross-reacted to 1% epoxy oligomer MW340 (which reacts with acrylic acid to form the epoxyacrylate), one to 1% bisphenol A and one to 0·5% acrylic acid, but none responded to methyl methacrylate. The booster dose of bisphenol A dimethacrylate increased the number of responders in the 20 animals by one only (from the original eight to nine). Six animals in this group, including one that did not react to the dimethacrylate, demonstrated cross-sensitization to the epoxy oligomer and two reacted to acrylic acid. No reactions to bisphenol A or methyl methacrylate were observed.

In the light of these findings, the epoxyacrylate should be classed as an extremely potent sensitizer and the dimethacrylate as a moderately active one. The epoxyacrylate has evidently also demonstrated activity in man; two men out of a total of six with dermatitis contracted from UV curing inks responded positively to it in a patch test. Neither of these responders cross-reacted to bisphenol A dimethacrylate. The investigators also make the important point that both of these modified prepolymers possess little or no irritant activity. The primary irritation index for the epoxyacrylate, as determined in the Draize test, was only 0·5, while that for the methacrylate was 1·7.

[The results of this study demonstrate that sensitizing ability cannot be inferred from the results of irritation studies. There is still some tendency in the ink industry to assume that a material that is a non-irritant cannot possibly damage the skin.]

Epoxide formation from styrene in man?

Pfäffli, P., Hesso, A., Vainio, H. & Hyvönen, M. (1981). 4-Vinylphenol excretion suggestive of arene oxide formation in workers occupationally exposed to styrene. *Toxic. appl. Pharmac.* **60,** 85.

Styrene-7,8-oxide (phenyl oxirane), a primary metabolite of styrene, is mutagenic both *in vitro* and *in vivo* (*Cited in F.C.T.* 1980, **18,** 434). Its formation from styrene in human lymphocyte cultures has

recently been demonstrated (Norppa *et al. Carcinogenesis* 1980, **1**, 357). However, in an Ames test with *Salmonella typhimurium* TA100 this metabolite could not completely account for the mutagenicity of metabolically-activated styrene, since the sum of the 7,8-oxide and its hydrolytic product 1-phenyl-1,2-ethanediol formed was much smaller than the amount that induced mutagenesis (Watabe *et al. Scand. J. Work envir. Hlth* 1978, **4**, Suppl. 2, 142). It was suspected that arene oxides might also play a role; one, styrene-3,4-oxide, was found to kill *S. typhimurium* TA100, TA98 and TA1537 in the absence of S-9 mix, but in the presence of S-9 it was a more potent mutagen to TA100 cells than was styrene. This suggested that an oxygenated metabolite of 1,2-dihydroxy-1,2-dihydro-4-vinylbenzene, which results from the 3,4-oxide under the action of epoxide hydratase in the S-9 mix, is a proximate mutagen (Watabe *et al. loc. cit.*). Indirect evidence for the formation of the 3,4-oxide has come from the detection of its isomeric product 4-vinylphenol (VP) in the urine of rats given styrene intraperitoneally (Pantarotto *et al. ibid* 1978, **4**, Suppl. 2, 67) or orally (*Cited in F.C.T.* 1971, **9**, 301) and Pfäffli *et al.* (cited above) now report its presence in human urine.

Urine samples were obtained from workers in two reinforced-plastics factories, who were engaged in rolling polyester plastics by hand or by spray application. The average air concentration of styrene was about 130 ppm, and the urine samples were collected at the end of the 8-hr workshift. Nine samples from one factory and seven and 13 from the other factory (collected on two occasions) contained mean levels of 0·06 ± 0·028, 0·027 ± 0·023 and 0·024 ± 0·017 nmol VP/g creatinine, respectively. The VP was detected by gas chromatography and gas chromatography–mass spectrometry. No VP above the analytical limit of detection of 0·001 mmol/litre was found in the urine of unexposed individuals. Mandelic acid (MA), which is derived from oxidation of the vinyl group, was also detected in the three groups of urine samples, at mean levels of 19·5 ± 10·4, 8·0 ± 5·7 and 7·5 ± 5·2 nmol/g creatinine respectively. There appeared to be a direct correlation between urinary levels of VP and MA, with little interindividual variation, the VP:MA ratio being in the range 0·31–0·34%.

These findings strongly suggested the intermediate formation of styrene-3,4-oxide in man, although pathways not involving arene oxide formation could not be excluded entirely. The much greater quantities of MA than of VP formed suggested that oxidation of the vinyl group (with the intermediate formation of the 7,8- rather than the 3,4-oxide) is quantitatively by far the most important route of styrene metabolism; however, some of the 3,4-oxide formed may not have been detectable as VP, due for example to the formation of glutathione conjugates or reaction with proteins or nucleic acids.

A lifetime of bis-chloromethyl ether

Leong, B. K. J., Kociba, R. J. & Jersey, G. C. (1981). A lifetime study of rats and mice exposed to vapors of bis(chloromethyl)ether. *Toxic. appl. Pharmac.* **58**, 269.

There have been many studies to examine the effects of chloromethyl methyl ether or its industrial contaminant bis-chloromethyl ether (BCME) in both experimental animals and humans (*Cited in F.C.T.* 1977, **15**, 244; *ibid* 1981, **19**, 275) and these have shown a variety of pathological changes in the respiratory tract. Several papers cited in an earlier review (*ibid* 1977, **15**, 245) reported the occurrence of respiratory and nasal tumours (aesthesioneuroepitheliomas) in animals exposed to only 1 ppm or 0·1 ppm BCME, in some cases for only a few days.

The present study sought to extend these investigations and find, if possible, a no-observed-effect level for BCME in rats and mice. Groups of 120 male specific-pathogen free (SPF) Sprague–Dawley rats or 144–157 male Ha/ICR mice were exposed in inhalation chambers to 100, 10 or 1 ppb BCME vapour in air. Groups of control rats and mice were kept under ambient conditions without chamber exposures. For each of the treatment groups, exposures were given for 6 hr/day, 5 days/wk for 6 months. Subsequently, observations were conducted for the duration of the animals' lifespans. Evaluation of groups of eight rats killed at the end of the 6-month exposure period revealed no abnormalities in haematology, exfoliate cytology of lung washes or cytogenetic parameters of bone-marrow cells, and no lesions that could be attributed to BCME exposure were found at autopsy.

However, 96/111 (86·5%) of the rats that had been exposed to 100 ppb BCME subsequently developed nasal tumours and pulmonary adenomas were observed in 4% of the group. The nasal tumours were visible grossly as a dorsal protruding mass on the bridge of the nose and on examination were generally found to extend into the cerebrum. A few had metastasized to regional lymph nodes and/or the lungs. The groups of rats exposed to 10 or 1 ppb BCME had none of these nasal or pulmonary tumours, and none were observed in the control group. A variety of non-respiratory tumours were seen in all groups, but these were not thought to be related to BCME exposure. The bronchogenic squamous cell carcinomas observed in an earlier study (Kuschner *et al. Archs envir. Hlth* 1975, **30**, 73; *Cited in F.C.T.* 1977, **15**, 245) were not seen in this study and this might indicate a strain difference in response to BCME between the SPF caesarian-derived and non-SPF Sprague–Dawley rats used in the two studies.

The mice exposed to 100 ppb BCME did not develop nasal tumours but those surviving beyond the 6-month exposure period showed a significant increase in the incidence of pulmonary adenomas over the control mice. This significance was not seen in the incidence of pulmonary adenomas over the entire study period. Although some lung tumours did occur in the mice given lower doses, no significant increase was seen at any time interval.

In both species, therefore, effects were seen at 100 ppb, and 10 ppb appears to be the no-observed-effect level for a 6-month exposure period. The median cancer induction time for rats exposed to 100 ppb BCME was found to be 350 days which is shorter than previously reported probably as a result of the earlier detection of nasal tumours. The other data are consistent with previous studies and confirm

the exposure-response curve of Kuschner *et al.* (*loc. cit.*).

Acrylamide neurotoxicity under the microscope

Burek, J. D., Albee, R. R., Beyer, J. E., Bell, T. J., Carreon, R. M., Morden, D. C., Wade, C. E., Hermann, E. A. & Gorzinski, S. J. (1980). Subchronic toxicity of acrylamide administered to rats in the drinking water followed by up to 144 days of recovery. *J. envir. Path. Toxicol.* **4** (5–6), 157.

When we last discussed the neurotoxicity of acrylamide (*Cited in F.C.T.* 1981, **19,** 395) we reported the results of a 13-wk study in Fischer rats. Although that study provided some useful information on the subchronic toxicity of the compound, it did not investigate fully the ultrastructural changes induced by acrylamide ingestion.

Such changes have been examined in the present study in which groups of ten female and 23–29 male Fischer rats were given 0, 0·05, 0·2, 1, 5 or 20 mg acrylamide/kg body weight/day in their drinking-water for about 90 days. Ten males in each dose group were kept, untreated, for a further 144 days to observe recovery. The onset of neuropathy was evaluated by the landing foot-spread (hind-limb splaying) method following a drop from a height of 12 in. (*ibid* 1978, **16,** 189). Autopsies were performed on 59 males and 60 females at the end of dosing and on four males from each dose group after the recovery period. Body weights were determined weekly, and blood and urine samples were taken during dosing and recovery. Nervous tissues were examined using light and electron microscopy.

The effects observed at the end of the treatment period in the highest dose group—hind-limb neuropathy and decreased body-weight gain—were similar to those seen in the previous study (*ibid* 1981, **19,** 395) but in addition atrophy of the skeletal muscle, testicular atrophy and distended urinary bladders were observed, possibly occurring as a result of nervous damage. After 144 days of recovery, the effects in the high-dose group were almost completely reversed. In the earlier study no adverse effects had been observed at the 5-mg/kg level but peripheral nerve degeneration was observed at this level in the present experiment. These effects were less marked than in the high-dose group and appeared to have reversed completely after 111 days of recovery. In rats given 1 mg/kg/day, a minimal treatment-related effect was observed in males at the end of the treatment period, and this was limited to very slight nerve degeneration observed under the electron microscope (nervous tissue from females was not examined by electron microscopy). The degeneration appeared to have reversed after 25 days of recovery. No treatment-related effects were observed in any of the rats given 0·05 or 0·2 mg/kg/day.

The study demonstrates the apparent reversibility of acrylamide neurotoxicity in the rat, even following a large toxic insult. In addition, acrylamide-induced nervous tissue toxicity does not appear to be a 'dying-back' neuropathy; in this study the nervous lesions were observed to be multifocal and involved an apparently random degeneration along individual axons. Some areas within a single axon were fragmented, some swollen, some had myelin loss and still others were normal. These changes were not found in a sequence, and apparently normal areas were often located between degenerative areas.

No teratogenicity from ethyl acrylate in rats

Murray, J. S., Miller, R. R., Deacon, M. M., Hanley, T. R., Jr, Hayes, W. C., Rao, K. S. & John, J. A. (1981). Teratological evaluation of inhaled ethyl acrylate in rats. *Toxic. appl. Pharmac.* **60,** 106.

Ethyl acrylate is used extensively in the manufacture of a variety of plastics and resins. Its toxicity is low following inhalation exposure in a number of animal species. No significant adverse effects were observed in rats exposed to 70 ppm ethyl acrylate for 7 hr/day for 30 days (Pozzani *et al. J. ind. Hyg. Toxicol.* 1949, **31,** 311) and exposure of rabbits, guinea-pigs and rats to 75 ppm for 50 7-hr periods over 72 days caused no ill-effects (Treon *et al. ibid* 1949, **31,** 317). However, the ACGIH has recently lowered its recommended TLV for ethyl acrylate for 25 to 5 ppm (*TLVs: Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Air with Intended Changes for 1981*, p. 18. ACGIH, Cincinnati, OH, 1981).

The authors cited above investigated the teratogenic potential of ethyl acrylate by exposing groups of 33 Sprague–Dawley rats, in inhalation chambers, to filtered room air, or to 50 or 150 ppm ethyl acrylate for 6 hr/day on days 6–15 of gestation. The rats were killed on day 21 of gestation. Signs of maternal toxicity, including decreased weight gain and food consumption and increased water consumption, were observed at the 150-ppm exposure level. Exposure to ethyl acrylate had no effect on mean litter size, the incidence of resorptions or the foetal sex ratio. The mean body weight of foetuses of dams exposed to 150 ppm ethyl acrylate was significantly increased, but the authors concluded that this was not toxicologically significant.

The incidence of major malformations was slightly but not significantly increased among foetuses in the 150-ppm exposure group. In this group three foetuses from three different litters had major malformations compared with none in either the lower dose group or the controls. A significant decrease in the incidence of delayed ossification of the cervical centra was observed in both of the groups exposed to ethyl acrylate compared with the controls, and there was a significantly lower number of foetuses with delayed ossification of the sternebrae in the group exposed at the higher level. However it was considered that these effects were not treatment related. The authors conclude on the basis of this study that inhalation of 50 or 150 ppm ethyl acrylate during the stage of major organogenesis is not teratogenic in the rat.

Benzoyl peroxide is a promoter in mice

Slaga, T. J., Klein-Szanto, A. J. P., Triplett, L. L., Yotti, L. P. & Trosko, J. E. (1981). Skin tumor-pro-

moting activity of benzoyl peroxide, a widely used free radical-generating compound. *Science, N.Y.* **213,** 1023.

Benzoyl peroxide, a strong oxidizing agent, is used as an additive in the rubber and plastic industries, as a bleaching agent for bread, flour, oils, fats and waxes, and in dermatological therapy in, for example, the treatment of mild acne. Until recently, positive toxic effects have been limited to moderate skin irritation and contact sensitization. While benzoyl peroxide has consistently given negative results as a complete carcinogen (*Cited in F.C.T.* 1963, **2,** 69; *ibid* 1965, **3,** 542; Sharratt *et al. Fd Cosmet. Toxicol.* 1964, **2,** 527), carcinogenic studies have widened to encompass promoters and initiators. A recent study carried out on mice has confirmed that benzoyl peroxide is not a complete skin carcinogen or a skin-tumour initiator but has demonstrated that it is an effective promoter of both papillomas and squamous cell carcinomas.

Five groups of 30 female Sencar mice received a single topical application of 10 nmol of the initiator 7,12-dimethylbenz[*a*]anthracene (DMBA) in acetone, followed by topical applications of 0, 1, 10, 20 or 40 mg benzoyl peroxide in acetone twice weekly for 52 wk. Five other groups were given one topical application of 0, 1, 10, 20 or 40 mg benzoyl peroxide, followed by applications of 2 μg of the promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) twice weekly for 52 wk. A third set of five groups received topical applications of 0, 1, 10, 20 or 40 mg benzoyl peroxide twice weekly for 52 wk. Benzoyl peroxide was not effective as a complete carcinogen and failed to show any tumour initiating activity when administered prior to TPA application. However, following initiation by DMBA, a significant number of papillomas and carcinomas were produced even at the lowest peroxide dose level.

Further morphological studies *in vivo* together with *in vitro* studies provided additional evidence of the tumour-promoting capacity of benzoyl peroxide. Similar activity has previously been demonstrated in a number of other compounds that generate free radicals. The authors discuss free radical-induced membrane changes that may be involved in the promoting activity of benzoyl peroxide.

Are dioxane and hexachlorobutadiene genotoxic carcinogens?

Stott, W. T., Quast, J. F. & Watanabe, P. G. (1981). Differentiation of the mechanisms of oncogenicity of 1,4-dioxane and 1,3-hexachlorobutadiene in the rat. *Toxic. appl. Pharmac.* **60,** 287.

1,4-Dioxane (DX) produced nasal and hepatocellular carcinomas in rats when given at 1% in their drinking-water for 2 yr, but at 0·1% there was only liver and kidney damage, and at 0·01% no untoward effects were observed. These dose levels were equivalent in the males to 1015, 94 and 9·6 mg/kg/day respectively (*Cited in F.C.T.* 1975, **13,** 674). A marked dose-response was similarly found when 1,3-hexachlorobutadiene (HCBD) was fed to rats for 2 yr; renal tubular adenomas and adenocarcinomas developed at 20 mg/kg/day, while at 2·0 mg/kg there was a possible increase in hyperplasia of the renal tubular epithelium and an adenomatous proliferation of this epithelium in females, and at 0·2 mg/kg there were no treatment-related effects (*ibid* 1979, **17,** 97). Whether DX and HCBD cause tumours in rats by a genetic or non-genetic mechanism has now been explored.

Male rats (4–5/group) were given DX either by gavage in a single dose of 10, 100 or 1000 mg/kg in saline or in the drinking-water at levels providing doses of 10 or 1000 mg/kg/day for 11 wk, while similar groups were given HCBD by gavage at 0·2 or 20 mg/kg in corn oil, singly or daily for 3 wk. To facilitate an assessment of DNA synthesis, indicative of cellular regeneration following damage, [6-^{3}H]thymidine was administered at least 6 hr before the acute treatments and on the last day of repeated administration. The animals were killed 7 days after thymidine administration and the livers of DX-treated rats and the kidneys of HCBD-treated rats were examined. DX given for 11 wk at 1000 mg/kg/day produced an increase in relative liver weights and a minimal degree of centrilobular hepatocellular swelling, accompanied by a significant (1·5-fold) increase in hepatic DNA synthesis, but no such changes occurred at other treatment levels. HCBD given at 20 mg/kg/day for 3 wk decreased body weight, increased relative kidney weight and caused histopathological changes in the renal tubular epithelial cells consistent with simultaneous tissue degeneration and regeneration. There was also a 1·8-fold increase in renal DNA synthesis, although because of large individual variation this was not statistically significant. In one of two acute experiments with HCBD, DNA synthesis was again increased at the 20-mg/kg level, but histology was not examined in this case. In contrast, the potent genotoxic carcinogen *N*-nitrosodimethylamine (NDMA) at a tumorigenic dose level of 3 mg/kg/day for 3 wk failed to affect body weight, relative liver and kidney weights or histopathology, and although DNA synthesis increased in the liver this was not to a statistically significant extent.

DNA alkylation was then measured in rats given ^{14}C-labelled DX, HCBD or NDMA (1000, 20 and 3 mg/kg respectively) by gavage, and killed 4 hr (DX and NDMA) or 6–30 hr (HCBD) later. DNA repair was also assessed in rats given the unlabelled compounds at 1000, 20 and 20 mg/kg, respectively, followed by repeated (1·5-hourly) ip injections of hydroxyurea to repress normal DNA synthesis, and hourly sc injections of [6-^{3}H]thymidine for 6 hr. DX had no detectable effect on either hepatic DNA repair or alkylation (the latter having a detection limit of one alkylation/10^6 nucleotides), whereas HCBD increased renal DNA repair 1·27- and 1·54-fold in two trials, and produced an average alkylation rate of 0·78/10^6 nucleotides. High-pressure liquid chromatography analysis of isolated nucleosides from the purified renal DNA of these rats revealed a small radioactive peak just preceding elution of deoxycytosine, the shape and position of which indicated a true HCBD-DNA alkylation product, rather than carbon-1 incorporation into normal DNA components or trace contamination with RNA or glycogen. HCBD was, however, far less potent than NDMA, which increased renal and hepatic DNA repair 2·87

and 3·72 times respectively and produced 23·8 alkylations/10^6 nucleotides in the kidney and 167 alkylations/10^6 nucleotides in the liver.

DX in a wide range of concentrations was not mutagenic in an Ames test with *Salmonella typhimurium* strains TA1535, 1538, 98 or 100, with or without metabolic activation, or in a rat primary hepatocyte unscheduled synthesis bioassay. HCBD also gave negative results in the latter test, and previous positive findings with *S. typhimurium* strain TA100 (Simmon, *Proc. 2nd FDA Office of Science Summer Symposium 1977*, pp. 163 & 171) could not be confirmed.

It was concluded that DX causes tumours by a non-genetic mechanism (possibly through repeated tissue injury when administered at cytotoxic dose levels, with the resultant cellular regeneration and increased DNA replication increasing the chance of spontaneous base errors). Exposure at non-cytotoxic levels should therefore pose no risk of carcinogenicity. HCBD may also be acting primarily by such a mechanism, but a minor genetic contribution cannot be discounted.

[It would have been valuable to extend these investigations to the nasal tissues of DX-treated rats, to establish that the nasal tumours too are the product of a non-genotoxic mechanism. It may also be significant that DX suppressed T-cell responses while augmenting B-cell responses in mouse lymphocytes *in vitro* (Thurman *et al. Toxic. appl. Pharmac.* 1978, **44,** 617); this suggests that immunosuppression could play a role in its carcinogenicity, by interfering with the normal mechanism for eliminating abnormal or malignant cells. Further research is also desirable into the possible role of the principal urinary metabolite of DX, *p*-dioxan-2-one, which is more acutely toxic than DX, and which has a chemical structure suggesting that it may be a proximate carcinogen (Woo *et al. Cancer Res.* 1978, **38,** 1621).]

The genotoxicity of MOCA and aniline

McQueen, C. A., Maslansky, C. J., Crescenzi, S. B. & Williams, G. M. (1981). The genotoxicity of 4,4′-methylenebis-2-chloroaniline in rat, mouse, and hamster hepatocytes. *Toxic. appl. Pharmac.* **58,** 231.

4,4′-Methylene-bis-(2-chloroaniline) (MOCA) is an established animal carcinogen, having produced tumours of the liver, lung, mammary and zymbal glands, and vascular system in rats, liver and vascular tumours in mice, and bladder tumours in dogs (*Cited in F.C.T.* 1973, **11,** 158; *ibid* 1976, **14,** 217: Kommineni *et al. J. envir. Path. Toxicol.* 1979, **2** (5), 149; Stula *et al. Toxic. appl. Pharmac.* 1975, **31,** 159; Stula *et al. J. envir. Path. Toxicol.* 1977, **1,** 31). More recently, aniline (as the hydrochloride) has also been shown to produce haemangiosarcomas of the spleen and fibrosarcomas and sarcomas of the spleen and other organs in rats (*Federal Register* 1978, **43,** 50741; *Chemical Industry Institute of Toxicology 1980 Annual Report and Scientific Review: Science in the Public Interest*). However, whereas MOCA gave positive results in bacterial mutation, cell-transformation, degranulation, and sebaceous-gland suppression tests, negative results were obtained with aniline in these and two other short-term predictive tests (Purchase *et al. Br. J. Cancer* 1978, **37,** 873). Other bacterial tests with aniline have also been almost universally negative (e.g. McCann *et al. Proc. natn. Acad. Sci. U.S.A.* 1975, **72,** 5135; Poirier & de Serres, *J. natn. Cancer Inst.* 1979, **62,** 919) although it did induce mutations in *Salmonella typhimurium* TA98 in the presence of norharman and S-9 mix (Nagao *et al. Proc. Japan Acad. Ser. B*, 1977, **53,** 34). In addition it produced sister-chromatid exchanges, but not chromosome aberrations, in cultured Chinese hamster cells (Abe & Sasaki, *J. natn. Cancer Inst.* 1977, **58,** 1635).

McQueen *et al.* (cited above) have now tested the genotoxicity of MOCA and aniline in hepatocytes isolated by *in vivo* perfusion of rat, mouse and hamster livers with collagenase. DNA repair, assessed by autoradiographic measurement of ^{3}H-labelled thymidine incorporation, was elicited in all species by MOCA. The amount of such repair was dose-dependent up to cytotoxic concentrations, reaching a maximum at 10^{-5} M in rat and hamster cells and 5×10^{-5} M in mouse cells. At these maxima, the number of grains per nucleus was 64 and 129 in two rat tests, 17 and 22 in two hamster tests and 59 and 73 in two mouse tests. However, aniline (tested as 10^{-5} M) was negative in all species.

[The test method used in this study appears to be a sensitive predictor of chemical carcinogenicity (*Long-term and Short-term Screening Assays for Carcinogens: A Critical Appraisal.* IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, supplement 2, p. 201. IARC, Lyon, 1980). It has provided further evidence that MOCA is a genotoxic carcinogen, thus adding support to recent UK measures to lessen the degree of industrial exposure to this compound (*Health and Safety: Manufacturing and Service Industries 1976; Health and Safety: Manufacturing and Service Industries 1978; Health and Safety Executive, London, 1978 and 1980, respectively*). However, aniline has proved negative in this as in most other short-term tests. Whether this indicates that its mechanism of action is epigenetic, or simply that the predictive value of many such tests is still far from perfect, has still to be elucidated. CIIT studies, demonstrating the covalent binding of aniline and/or its metabolites to rat red-blood cells and spleen (Sun & Bus, *Pharmacologist* 1980, **22,** 207) and the induction of sister-chromatid exchanges in human fibroblasts by the metabolites phenylhydroxylamine and *o*-aminophenol (*CIIT Activities* 1981, **1** (4), 3) would tend to support the latter hypothesis.]

Immunotoxicity of OPP and Fyrol FR2 explored

Luster, M. I., Dean, J. H., Boorman, G. A., Archer, D. L., Lauer, L., Lawson, L. D., Moore, J. A. & Wilson, R. E. (1981). The effects of orthophenylphenol, tris(2,3-dichloropropyl) phosphate, and cyclophosphamide on the immune system and host susceptibility of mice following subchronic exposure. *Toxic. appl. Pharmac.* **58,** 252.

An increasing number of chemicals are now recognized to affect immunological function. *o*-Phenylphenol (2-hydroxybiphenyl; OPP), which is used as a pre-

servative on citrus fruit, was in 1979 reported to depress the antibody plaque-forming cell responses in 12–20-wk-old male mice when 1 or 10 ppm was included in their drinking-water for 4 wk or more (La Via & La Via, *Drug Chem. Toxicol.* 1979, **2,** 167). This contrasted with earlier reports indicating a low acute and chronic toxicity in rats and dogs, on the basis of which JECFA allocated unconditional and conditional ADIs of up to 0·2 and 1·0 mg/kg, respectively (*Cited in F.C.T.* 1965, **3,** 812 & 819). The immunological effects of the flame retardant tris (2,3-dichloropropyl) phosphate (Fyrol FR2) have not previously been investigated. However, the parent compound and two of its predicted metabolites were mutagenic in an Ames test (*ibid* 1979, **17,** 314). Moreover, 1,3-dichloro-2-propanone, which was the most potent predicted metabolite in this respect, has the structure of an alkylating agent and hence is a suspected carcinogen and immunosuppressant. The immunotoxicity of OPP and Fyrol FR2 in mice has now been studied, using cyclophosphamide (CY), a therapeutically-employed alkylating agent with established immunosuppressive effects (Dean *et al. Drug Chem. Toxicol.* 1979, **2,** 133) as a positive control.

Female mice aged 6–8 wk were given OPP by gavage in corn oil at 1, 10 or 200 mg/kg body weight/day for 10 days over 2 wk, Fyrol FR2 (also in corn oil) at 0·25, 2·5 or 25 mg/kg body weight by sc injection for 4 days, or CY at 45 mg/kg body weight by ip injection for 4 days. All animals were subjected to measurement of body and organ weights, investigations of haematology, clinical chemistry and histopathology, and a variety of tests of immunological function and host susceptibility. These involved studies of bone-marrow cellularity and colony formation, splenic lymphoproliferative responses to the T-cell mitogens phytohaemagglutinin and conconavalin A and the B-cell mitogen *Escherichia coli* lipopolysaccharide, delayed hypersensitivity responses to keyhole-limpet haemocyanin, serum immunoglobulin (IgG, IgM and IgA) concentrations, *in vivo* and *in vitro* antibody responses to sheep red blood cells and other agents, mortality after injection of *Listeria monocytogenes*, and tumour development after injection of polyoma tumour cells.

OPP at the highest level slightly increased relative thymus and spleen weights and serum cholesterol levels and decreased serum triglycerides and the albumin:globulin ratio, but had no significant immunosuppressive effects. A level of 100 μg/culture added to mouse spleen cells *in vitro* suppressed antibody responses, but this was due to cell lysis rather than to inhibition of lymphocyte function. The highest level of Fyrol FR2 caused lymphoid depletion of the thymus in 20% of mice, increased serum cholesterol and decreased serum triglycerides, while the albumin:globulin ratio was decreased at the two lower levels. The lymphoproliferative response to conconavalin A and to lipopolysaccharide was depressed at 25 mg/kg/day and the response to the latter was also depressed at 10 mg/kg/day. In addition there was an increased tumour incidence after injection of tumour cells, in mice given 25 mg Fyrol FR2/kg/day. In contrast, CY produced thymic atrophy in all of the mice, severe leucopaennia, bone-marrow hypocellularity and disturbed liver function, and had marked effects in all tests of immunological function and host susceptibility.

It was concluded that OPP lacked immunotoxicity under the conditions of the study, and that Fyrol FR2 was only weakly active. In the study on OPP by La Via & La Via (*loc. cit.*), which yielded conflicting results, mice of a different sex, strain and age were used and administration of OPP was in the drinking-water rather than by gavage. However, the precise reason for the differing results is unknown.

5-Methoxypsoralen gets a tanning

Zajdela, F. & Bisagni, E. (1981). 5-Methoxypsoralen, the melanogenic additive in sun-tan preparations, is tumorigenic in mice exposed to 365 nm u.v. radiation. *Carcinogenesis* **2,** 121.

5-Methoxypsoralen (5-MOP) is naturally present in the oils of angelica seeds and roots, in parsley and celery leaves and in the volatile oils from the peels of lime and bergamot. As a group, the psoralens have strong skin-photosensitizing properties, and induce hyperpigmentation in the presence of UV light. A combination of a psoralen and ultraviolet A radiation (PUVA therapy) is highly effective in the treatment of psoriasis but results in an increased risk of skin cancer. It has been suggested that the use of 5-MOP as a tanning promoter in commercially available sun-tan preparations may lead to an increased risk of cancer although this risk may be countered by the protection against the carcinogenic UVB rays from the sun offered by more rapid tanning. The degree of hazard resulting from the use of sun-tan preparations containing 5-MOP remains uncertain as much of the evidence put forward hinges on the results of unpublished studies (*Cited in F.C.T.* 1981, **19,** 270). A mouse skin carcinogenicity study has now been reported in which the activities of 5-MOP, 8-MOP and psoralen were compared.

Groups of 40 albino mice aged 12–14 wk were treated with 5-MOP, 8-MOP or psoralen five times a week for 23 wk. The compounds were applied to the ears as a 0·25% solution in acetone (40 μl; 10 μg 5-MOP, 8-MOP or psoralen/cm^2). The acetone was allowed to evaporate and the animals were subjected to 365 nm UV radiation. (The dose of psoralen that would, in combination with UVA, cause tumours in nearly 100% of the animals was determined and this dose was used to test the relative carcinogenicity of the three compounds.) Half of the 5-MOP-treated animals were given, 8 months after the last 5-MOP application, 40 promotional treatments of 1 μg of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)/ear, three times a week. All of the animals were then observed for as long as possible.

For the psoralen, 8-MOP, 5-MOP and 5-MOP + TPA treatments, the proportions of animals developing tumours were 97, 92, 85 and 100% respectively. The figures for animals having multiple tumours were 50, 45, 25 and 60% respectively. Tumours caused by 5-MOP showed much longer latent periods (12–21 months after start of treatment) than those induced by psoralen (7–13 months), while those induced by 8-MOP were intermediate (10–18

months). Most of the tumours that developed were squamous-cell carcinomas of the moderately differentially keratinizing type, the majority exceeding a volume of 500 mm^3 at autopsy.

The authors concluded that 5-MOP tested in albino mice by topical application has a carcinogenic activity close to that of psoralen and 8-MOP, that cells initiated by 5-MOP can remain present for a long time in treated tissue, and that it is probable that initiation lesions in DNA can accumulate over a period of time.

Don't touch the water

Greaves, M. W., Black, A. K., Eady, R. A. J. & Coutts, A. (1981). Aquagenic pruritis. *Br. med. J.* **282,** 2008.

We have reported in these pages many unusual allergies. This, however, must count as one of the most unexpected, and yet it may prove to be quite common—an allergy to water.

The authors, from the Institute of Dermatology, St John's Hospital for Diseases of the Skin in London, describe three patients who complained of intense itching following even brief contact with water at any temperature. Bathing resulted in irritation which started locally and then became more generalized, lasting up to 2 hr in one patient. Blockade of sweating by topical pretreatment of a selected area of skin with 3% hyoscine in two of the patients prevented local itching although the surrounding areas itched. Cimetidine (200 mg) and chloropheniramine (8 mg) given every 6 hr for 24 hr in the same two patients completely prevented itching. One of these patients subsequently found that chlorpheniramine taken 2 hr before bathing was sufficient to prevent the itching while the other required both drugs on a strict daily regime. [The effect of antihistamines was not studied fully in the third patient.]

The patients were otherwise apparently healthy and had often been referred to as 'neurotic'. However, pharmacological studies showed that the condition was associated with an abnormally high percentage of degranulated skin mast cells before challenge with water, which increased further after challenge, and with raised blood histamine concentrations which in two of the patients were unusually high even before immersion in water. Despite this clear increase in histamine in the skin, no visible changes were observed and only one patient demonstrated any systemic symptoms (dizziness and palpitations).

The pathogensis of this disorder is not clear and the authors report that they do not know of any previous case studies although they are aware of other irritant reactions in response to the contact of water with the skin such as cold, cholinergic or aquagenic urticaria. It is known that certain pathological conditions may be aggravated by prolonged exposure to water, and the existence of warm-water dermatitis is recognized (*Cited in F.C.T.* 1978, **16,** 402). However, the authors suggest that the condition described in the present paper, which they term aquagenic pruritis, is probably quite common since a number of their colleagues at the Institute recollect seeing patients with similar symptoms in the past.

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MEETING REPORT

TOXICOLOGICAL ASPECTS OF AGEING

When the British Toxicology Society (BTS) discussed ageing in the context of toxicology at its meeting at the University of Kent, Canterbury on 17 and 18 September 1981, it quickly became apparent that the subject is broad, diffuse and urgently in need of definitions and structure. Dr D. A. Hall of the Department of Medicine in Leeds defined ageing as "a decrease in ability to cope with the stresses of life". This is no doubt applicable to the inmate of a geriatric unit, but it is not true for the individual who merely aged his skin by excessive exposure to the sun or for the sedentary worker who is unaware of the fact that his vital capacity has fallen to half what it was in his youth. Nor is it helpful to the toxicologist who seeks ways of measuring ageing in various tissues and models for studying mechanisms involved in its pathogenesis. Are ageing changes genetically programmed or do they simply represent an accumulation of random damage due to exposure to environmental toxins? In general, as with most diseases, genetic and environmental factors interact as determinants of ageing effects. Certainly changes such as those that occur at and after the menopause in women are genetically programmed, but there are plenty of examples of facilitation of ageing processes by environmental factors such as smoking and sunlight.

It is all too easy to confuse an age-associated disease with ageing. In practice most chronic diseases if untreated get worse with time and hence with age. Pulmonary tuberculosis is an obvious example. An important first step, therefore, must be to try to distinguish between diseases that are primarily ageing phenomena and diseases that just happen to be more debilitating in old age but are not primarily due to ageing. In the present state of our knowledge it would seem reasonable to class post-menopausal osteoporosis as primarily an ageing phenomenon, and at the BTS meeting Dr J. Aaron from the Mineral Metabolism Unit in Leeds described the pathogenesis of osteoporosis in this light. Similarly, Dr Stephen Webster (Chesterton Hospital, Cambridge) described how the villi of the small bowel show progressive atrophy with age with the result that xylose absorption, which is customarily used to measure small-bowel function, is deficient in about 25% of old people.

It is now well established for laboratory animals and with less certainty for humans that diet restriction, especially early in life, is associated with increased longevity, with a reduced age-standardized risk of a variety of age-associated diseases and with a strikingly reduced risk of tumour development. This is an obvious starting point for active research into the field of ageing. A second line of approach relates to the study of age-related hormonal changes, particularly to ascertain the extent to which these are causes or effects of ageing.

It was particularly noticeable at the meeting that there is at present a serious paucity of laboratory animal models for the kinds of ageing effect that commonly present problems in man. For instance, there was no mention of animal models for studying senile osteoporosis or dementia. Half jokingly, no doubt, Dr Kenneth McCullagh (Searle Laboratories, High Wycombe) suggested that the elephant might be a good model for human atherosclerosis, but it hardly seems to be a practical alternative to the monkey or the White Carneau pigeon, neither of which is of more than limited value in this regard.

Professor Paul Grasso (British Petroleum, Sunbury) discussed the significance of lipofuscin pigment as an index of toxic damage. It is likely that in this case what happens in laboratory animals also occurs in man. But accumulation of pigment is not itself disabling and, therefore, is not of immediate interest to geriatricians.

Not unexpectedly, old people tend, for a long list of reasons including differences in pharmacokinetic profile and receptor sensitivity, to be more or less sensitive to various drugs than younger subjects. However, according to Dr P. Crome (New Cross Hospital, London) and Professor Ian Stevenson (University of Dundee) no single pattern is involved and each drug must be considered individually in judging how to take a patient's age into account when prescribing. From the discussion on this subject, one was certainly not left with the impression that, in future, safety tests on drugs should include studies on old animals as well as young ones.

The toxicology of ageing is going to become an increasingly important subject. Clearly, therefore, it now needs to be put on to a firmer and more structured scientific basis than was apparent at the BTS meeting.

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MEETING ANNOUNCEMENTS

OCCUPATIONAL SAFETY AND HEALTH

An "International Symposium on Collaboration in the Transfer of Information in Occupational Safety and Health" is to be held at the Institute for Occupational Safety and Health Documentation "Edvard Kardelj", Niš, Yugoslavia on 1–2 June 1982. Amongst other topics to be considered are information collection and retrieval, including the use of computers, the preparation of bibliographies of references and gathering specific data, and training users of health and safety information tc be aware of its importance and to benefit from its accessibility. Further details may be obtained from Kocić Dragica, Secretary of the Organizational Committee, Institute for Occupational Safety and Health Documentation "Edvard Kardelj", 18000 Niš, Višegradska 33, pp. 141, Yugoslavia (Telex 16284 YU INSDOK).

HEALTH EFFECTS OF ESSENTIAL AND TOXIC ELEMENTS

A symposium on the health effects and interactions of essential and toxic elements is being organized by the Research Department, University Hospital, Lund and the Unit for Community Health Sciences, Dalby. It will be held in Lund, Sweden on 28 June–2 July 1982, and further information is available from Dr M. Aboulla, Research Department 2, EB-b1., University Hospital, S-221 85 Lund, Sweden.

CONFERENCE AND EXHIBITION ON MICROSCOPY

The Royal Microscopical Society's "Micro 82 International Symposia and Exhibition" will take place at the Bloomsbury Centre Hotel, Coram Street, London WC1 on 12–16 July 1982. The Symposia will include sessions on the microscopy of food and the use of microscopy in medical and industrial laboratories. At the trade exhibition it is expected that 40 or more companies will exhibit light and electron microscopes and ancillary equipment. Further information, tickets for the exhibition and symposia registration forms are available from The Royal Microscopical Society, 37/38 St Clements, Oxford OX4 1AJ.

SYMPOSIUM ON IMMUNOTOXICOLOGY

A preliminary announcement has been made regarding an international symposium on immunotoxicology to be held at the University of Surrey on 13–17 September 1982. The symposium will include sessions on the effects of chemicals on the immune system, chemical-induced hypersensitivity, immune-mediated chemical toxicity and animal models for studying the effects of chemicals in the immune system. Further details can be obtained from Mrs J. McCall, Conference Secretary, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH.

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FORTHCOMING PAPERS

It is hoped to publish the following research papers and other communications in the next issue of *Food and Chemical Toxicology:*

Short-term toxicity study of metatartaric acid in rats. By A. J. Ingram, K. R. Butterworth, I. R. Gaunt and S. D. Gangolli.

Mutagens from the cooking of food II. Survey by Ames/Salmonella test of mutagen formation in the major protein-rich foods of the American diet. By L. F. Bjeldanes, M. M. Morris, J. S. Felton, S. Healy, D. Stuermer, P. Berry, H. Timourian and F. T. Hatch.

Mutagens from the cooking of food III. Survey by Ames/Salmonella test of mutagen formation in secondary sources of cooked dietary protein. By L. F. Bjeldanes, M. M. Morris, J. S. Felton, S. Healy, D. Stuermer, P. Berry, H. Timourian and F. T. Hatch.

Mutagenicity of the products obtained from heated milk systems. By A. N. Rogers and T. Shibamoto.

The mutagenicity of some edible mushrooms in the Ames test. By A. von Wright, J. Knuutinen, S. Lindroth, M. Pellinen, K.-G. Widén and E.-L. Seppä.

Nitrate reductase and nitrite levels in saliva of various habit groups. By U.S. Murdia, F. J. Mehta and S. V. Bhide.

Reduction of teratogenic effects of ethylenethiourea in rats by interaction with sodium nitrite *in vivo*. By K. S. Khera.

Hepatic effects of R-goitrin in Sprague–Dawley rats. By K. Nishie and M. E. Daxenbichler.

Isoflavone content of soya-based laboratory animal diets. By P. A. Murphy, E. Farmakalidis and L. D. Johnson.

Patulin mycotoxicosis in the rat: toxicology, pathology and clinical pathology. By E. R. McKinley, W. W. Carlton and G. D. Boon.

Failure to produce hypertension in rats by chronic exposure to cadmium. By H. Fingerle, G. Fischer and H. G. Classen.

Hepatic microsomal enzyme induction by Aroclors 1248 and 1254 in Cynomolgus monkeys. By F. Iverson, J. Truelove and S. L. Hierlihy.

Acute oral toxicity of inorganic cobalt compounds in rats. By G. J. A. Speijers, E. I. Krajnc, J. M. Berkvens and M. J. van Logten. (Short Paper).

Speculations on an extended dose-response model for carcinogenesis. By F. W. Carlborg. (Review Paper).

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Instructions to Authors

General. Original papers, reviews and letters to the editor may be submitted for publication. Manuscripts from the USA and Canada should be sent to the American Editor, Dr L. Golberg. All others must be sent to the Assistant Editor or to the appropriate Regional Editor. Letters must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Chemical Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Abstract: 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. Guppy, L., Lowes, N. R. & Walker, M. J. A. (1981). Effect of a proprietary rubefacient "Tiger Balm" on rabbit skin. *Fd Chem. Toxic.* **20,** 89.

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.

When reference to a paper is first made in the text the names of all authors should be given unless there are five or more, in which case the names of the first three should be given followed by the words *et al.* 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; Malinow, McNulty, McLaughlin *et al.* 1981); (McLaughlin *et al.* 1963; Malinow *et al.* 1981).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b, etc. following the citation of the year: e.g. 1943a, 1943b or (1943a,b).

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Chemical Nomenclature. The fundamental principles of organic and inorganic chemical nomenclature are laid down in the I.U.P.A.C. 1957 Rules (Butterworths Scientific Publications, London, 1958, 1959). These are given in *Handbook for Chemical Society Authors* (1961), pp. 16–163.

Other Nomenclature, Symbols and Abbreviations. In general, authors should follow the recommendations published in the *Handbook for Chemical Society Authors* (1961), p. 164 and in the *I.U.P.A.C. Information Bulletin*, No. 13, p. 64, Appendix B (1961). In the Title and Summary, abbreviations should be avoided; in the Introduction, Results and Discussion they should be used sparingly.

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